Technische Universität Braunschweig

Environmental Chemical Analysis Related to Drug Industries

Fate Monitoring of Diclofenac in Water/Sediment Systems

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

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eingereicht am: 18.10.2010
mündliche Prüfung (Disputation) am: 08.12.2010

Druckjahr 2010
Dissertation an der Technischen Universität Braunschweig,
Fakultät für Lebenswissenschaften

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeitraege

1- Mohamad, A., Bloem, E., Schnug, E., Kreuzig, R. (2009): Identification of phototransformation products of diclofenac in water and water/sediment systems. (Oral presentation) The annual conference of the German Chemical Society (GDCh: Gesellschaft Deutscher Chemiker) Environmental chemistry and ecotoxicology group "Behavior and Effects of Substances on the Environment'' Trier, Germany, 23-25 September, 2009.

2- Mohamad, A., Kreuzig, R., Bahadir, M. (2010): Fate monitoring of ^{14}C-diclofenac in laboratory water/sediment systems. (Oral presentation) The Annual Conference of International Association of Environmental Analytical Chemistry (IAEAC): 36th International Symposium on Environmental Analytical Chemistry, Rome, Italy, 5–9 October, 2010.

Acknowledgments

*First of all, whole praise is to **ALLAH**, the lord of the world, the source of all knowledge, the knower of everything, and who pleased me to finish this work during the period from 2006 to 2010 at the Institute of Environmental and Sustainable Chemistry, TU Braunschweig, Germany.*

*Great thanks to the **Egyptian government** for supporting me financially during the study period.*

*Special thanks to **Prof. Dr. mult. Dr. h.c. Müfit Bahadir**, the director of the Institute of Environmental and Sustainable Chemistry, TU Braunschweig, Germany, for accepting me as a Ph.D. candidate and providing me with all materials, chemicals, equipment and other consumable needed during the time of research.*

*From the depth of my heart, I am greatly indebted to my supervisor **apl. Prof. Dr. Robert Kreuzig** for devoting his time to follow my work almost day by day with his valuable comments, ideas, and guidance as well as for his encouragement, patience, and interest throughout the course of the study.*

*It is my pleasure to express my appreciation and thanks to **Prof. Dr. Hermann Wätzig,** Head of the division Pharmaceutical Analysis, Institute of Pharmaceutical Chemistry, TU Braunschweig, for accepting to be the chairman of the committee members of examination.*

Many thanks to ***Prof. Dr. Dr. Ewald Schnug*** *and* ***Dr. Elke Bloem****, Institute of Crop and Soil Science, Julius Kühn-Institut, for letting me use their LC/MS/MS instrument, to* ***Prof. Dr. Thomas Lindel****, and* ***Mrs Brigitte Brinkmann****, Institute of Organic Chemistry, TU Braunschweig, for supporting preparative column chromatography and semi-preparative HPLC analysis, to* ***Prof. Dr. Ludger Ernst****, head of NMR laboratories of the Chemical Institutes, TU Braunschweig, for supporting the NMR analysis and to* ***Sylvia Moenickes****, Institute of Geoecology, TU Braunschweig, for supporting the calculation of DT values.*

I would like also to express my deepest thanks to ***Dr. Sibylla Höltge*** *for the demonstration of the radiotracer techniques during the initial preparation of this work, and to* ***Dr. Roland Vogt*** *for his assistance, whenever necessary.*

I wish to convey my gratitude to my colleagues for providing a good working atmosphere, and for sharing their scientific knowledge.

Finally, I am deeply thankful and profoundly grateful to my parents, my wife, my sons (Seifeldin and Mohamed), my daughter (Lena), my sister and my brother for their love, care, pray, support, patience, and encouragement during the study period. Without them, I would have never the chance to achieve lot of things.

Dedication

to my

Parents, Wife,

Kids, Sister and

Brother

CONTENTS

Abbreviations

2D	Two dimensional
APCI	Atmospheric pressure chemical ionization
amu	Atomic mass unit
AOP	Advanced oxidation process
AP	Aqueous phase
Bq	Becquerel
BS	Laboratory-batch system
^{13}C-NMR	^{13}Carbon nuclear magnetic resonance
CAD	Collision affected dissociation
CAS	Chemical Abstracts Service
CE	Collision cell energy
CI	Chemical ionization
CID	Collision induced dissociation
CUR	Curtain gas
CXP	Collision cell exit potential
d	Day
DAA	Days after application
DCF	Diclofenac
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DP	Declustering potential
dpm	Decays per minute
ds	Dry substance
DT	Dissipation time
DT_{50}	Disappearance time in days for 50 % of the initially applied substance
DW	Drinking water
EC_{50}	Effective concentration required to induce a 50 % effect
Eh	Redox potential
EI	Electron impact
EI	Electrospray ionization
EMEA	European Medicines Agency
EMS	Enhanced full mass scan

EP	Entrance potential
EPI	Enhanced product ion scan
ER	Extractable residues
ERA	Environmental risk assessment
ESI	Electrospray ionization
EU	European Union
FAL	Federal Agriculture Research Center
FIA	Flow injection analysis
Gas 1	Nebulizer gas
Gas 2	Vaporizer gas
GC	Gas chromatography
GPC	Gel permeation chromatography
h	Hour
^{1}H-NMR	Proton nuclear magnetic resonance
Hg	Mercury
HLB	Hydrophilic lipophilic balance
hmbc	Heteronuclear multiple bond coherence
HPLC	High performance liquid chromatography
hsqc	Heteronuclear single quantum coherence
IAR	Initial applied radioactivity
IDA	Information depending acquisition
ihe	Interface heater
IR	Infrared
IS	Ion spray voltage
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
K_{biol}	Constant of biological degradation
K_d	Sorption or distribution coefficient
K_{des}	Desorption coefficient
K_F	Freundlich sorption coefficient
$K_{F\text{-}des}$	Freundlich desorption coefficient
K_{OC}	Distribution coefficient standardized on the organic substance
LC	Liquid chromatography
LD_{50}	Lethal dose required to kill a 50 % of the tested organisms
LSC	Liquid scintillation counter
m/z	Mass to charge ratio

MA	Method of analysis
MAX	Mixed-mode polymeric with reverse-phase and anion-exchange
MBq	Mega becquerel
MEC	Measured environmental concentration
MIN	Mineralization
min	Minute
MRM	Multiple reaction monitoring
MS	Mass spectrometry
ms	Milli second
MS/MS	Tandem mass spectrometry
mV	Milli volt
nd	Not detected
NER	Non-extractable residues
No.	Number
NOEC	No observed effect concentration
NSAIDs	Non-steroidal anti-inflammatory drugs
OECD	Organization for Economic Cooperation and Development
p	Person
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
ppm	Parts per million
Prec	Precursor ion scan
PTFE	Polytetrafluoroethylene
PTP	Phototransformation product
Qq	Quadrupole
QqQ	Triple quadrupole
QS	Quartz sand
R_f	Retention factor
RP	Reversed phase
rpm	Round per minute
RTLC	Radio thin layer chromatography
S1	Sandy sediment
S2	Sandy loam sediment
S3	Sandy loam sediment
SD	Standard deviation
SETAC	Society of Environmental Toxicology and Chemistry

SIR	Substrate induced respiration
SLT	Biometric flask equipped with soda lime trap
SOM	Solid organic matter
SPE	Solid phase extraction
SS	Suspended solids concentration
STP	Sewage treatment plant
STWW	Secondary-treated wastewater
SW	Surface water
t	Tons
TEM	Temperature
TGD	Technical guidance documents
TLC	Thin layer chromatography
TOC	Total oxygen content
TOF	Time of flight
TP	Transformation products
UV	Ultra violet
WHC	Water holding capacity
WHO	World Health Organisation
WS1	Water/sandy sediment
WS2	Water/sandy loam sediment
WS3	Water/sandy loam sediment
WWE	Wastewater effluent
WWI	Wastewater influent
WWTP	Wastewater treatment plant
y	year

1. Introduction

The daily human needs are many and different. Other than food and water, the need for medication is considered the most important thing at all. In the last decades, several diseases have been discovered and correspondingly several human pharmaceutical products for diagnosis, treatment or prevention of diseases have been discovered as well. Thus, 1200-3000 pharmaceutical compounds with different chemical structures are used worldwide in human and veterinary medicine [Halling-Sørensen et al., 1997, Hilton et al., 2003, Ashton et al., 2004, Carlsson et al., 2006a]. These pharmaceuticals, in some countries, are formulated in more than 7000 human and 1000 veterinary medicinal products [Carlsson et al., 2006a, Barron et al., 2009].

Humans are using these pharmaceuticals to get benefits without paying attention to the consequence of the uncontrolled emission of these pharmaceuticals into the environment. After excretion, unchanged parent compounds or formed metabolites are mainly introduced together with municipal wastewater into wastewater treatment plants for elimination purposes. A large number of these pharmaceuticals are polar and neither volatile nor biodegradable. In wastewater treatment plants, therefore, they are able to escape sorption onto the sewage sludge fraction, sedimentation and biotransformation during the activated sludge treatment in the aeration tank [Paxeus, 2004]. As a result, pharmaceuticals are released via wastewater effluents into receiving waters. Additionally, because pharmaceuticals are designed to persist long enough in the human body to perform their therapeutic effect, it is expected that they may remain in the aquatic environment for a specific time period according to the environmental conditions.

Although the consequence of presence of pharmaceuticals in the aquatic environment is not completely known, it should not be forgotten that they are biologically active substances, made for living organisms and hence may represent a danger for the ecosystem. Bioaccumulation [Halling-Sørensen et al., 1998, Brooks et al., 2005], additive or synergetic effects of complex mixtures substance [Cleuvers, 2003, Brian et al., 2005], endocrine disrupting activity [Halling-Sørensen et al., 1998, Brian et al., 2005, WHO 2002, Kime and Nash, 1999], phytotoxicity [Brain et al. 2004, Schmitt-Jansen., 2007] and the development of bacterial antibiotic resistance [Duong et al., 2008] as well as genotoxicity [Hartmann et al., 1998] are considered the most important harmful effects reported at present. Additionally, in water/sediment system, sediments may act as sinks for pharmaceuticals. Later on, under changed boundary environmental conditions, e.g., enhanced hydrological potentials or

changes from aerobic to anaerobic conditions, pharmaceutical residues may undergo bank filtration followed by entering into groundwater used for drinking water.

First studies that identified pharmaceuticals in the environment were published by Garrison et al. (1976) and Higaite and Azaznoff (1977) where clofibrate, aspirin and their metabolites were discovered in the effluent of a sewage disposal plant in USA. Afterwards, pharmaceuticals were detected in UK aquatic environment [Waggot, 1981, Richardson and Bowron, 1985]. The systematic research has been started in Germany at the beginning of the 1990s for determination of pharmaceuticals in rivers and wastewater treatment plants [Stumpf et al., 1996, Heberer and Stan, 1997, Heberer et al., 1997, 1998 and 1999, Ternes, 1998, and Hirsch et al., 1998, 1999, 2000]. Since this time, pharmaceuticals have been identified as a new class of environmental chemicals. Thereafter, the excessively increased use of pharmaceuticals allowed dispersing widely in the environment resulting in the detection of more than 80 pharmaceuticals of different therapeutic classes such as analgesics, antipyretics, human and veterinary antibiotics, antiepileptics, anti-inflammatories, antidepressants, beta-blockers, broncholitics, contraceptives, diagnostics, lipid regulators, synthetic steroidal hormones, tranquilizers and some of their related metabolites in different countries including Australia [Braga et al., 2005, Al-Rifai et al., 2007], Brazil [Stumpf et al., 1999], Canada [Metcalfe et al., 2003], Chinese [Anon, 2005], Finland [Vieno et al., 2005], France [Elbaz-Poulichet et al., 2002, Andreozzi et al., 2003, Comoretto and Chiron 2005, Rabiet et al., 2006], Germany [Ternes and Hirsch, 2000, Sacher et al., 1998, 2001, 2002, Zuehlke et al., 2004, Wiegel et al., 2004], Greece [Andreozzi et al., 2003, Koutsouba et al., 2003], Italy [Castiglioni et al., 2004, 2005, 2006, Zuccato et al., 2005], Japan [Nozaki et al., 2000, Kimura et al., 2007], Netherlands [Belfroid et al., 1999], Pakistan [Scheurell et al., 2009], Slovenia [Antonic and Heath 2007], Spain [Farre et al., 2001, Gomez et al., 2006], Sweden [Andreozzi et al., 2003], Switzerland [Buser et al., 1998a, b, Oellers et al., 2001, Golet et al., 2001, 2002, McArdell et al., 2003, Tixier et al., 2003, Tauxe-Wuersch et al., 2005], UK [Hilton and Thomas, 2003], and USA [Kolpin et al., 2002, Boyd et al., 2003].

Moreover, the pharmaceutical loads in a small Mediterranean river were found to be as important as pesticide loads [Comoretto and Chiron, 2005]. However, in contrast to pesticides, pharmaceuticals are used through the whole year meaning that they are continuously introduced into the environment [Castiglioni et al., 2006, Buchberger, 2007, Vieno et al., 2007]. Therefore, beside pesticides, heavy metals and other industrial chemicals, the environmental risk caused by pharmaceuticals has received increasing attention by several auothers [Halling-Sørensen et al., 1998, Stuer-Lauridsen et al., 2000,

Jones et al., 2001, 2002, Golet et al., 2002, Van Wezel and Jager, 2002, Carlsson et al., 2006a, b, Letzel et al., 2007, Schmitt-Jansen et al., 2007, Triebskorn et al., 2007, Grung et al., 2008, Huschek et al., 2008, Khalaf et al., 2009]. Recently, occurrence and effects of human pharmaceuticals in the aquatic environment are considered relevant by the regulatory procedure [EMEA, 2001, Roennefahrt et al., 2002]. The authorization of pharmaceutical groups has to be combined with environmental risk assessment and/or risk reduction measures [Grung et al., 2008]. The risk assessment of novel pharmaceuticals consists of two phases in which the second phase is consisted of two tiers **[Figure 1]**. In phase I, the trigger PEC_{water} > 0.01 µg/L has been established on the basis of an exposure assessment in surface water. If this trigger is exceeded, substances have to undergo extended Phase II tests. For critical substances, e.g., cytostatica and hormones, those tests are mandatory. In Tier A, fate studies and acute ecotoxicological tests in wastewater treatment plants and surface water have to be conducted. In Tier B, chronical effects, bioaccumulations, transformation, sorption onto soils are to be in the focus of further investigations. In Germany, the Federal Environment Agency has been determined the environmental impact of 136 veterinary and 134 human pharmaceuticals [Adler et al., 2008].

Among pharmaceuticals reported in the literature, non-steroidal anti-inflammatory drugs (NSAIDs) were found to gain a special relevance from being the most frequently mentioned environmental contaminants [Kümmerer, 2004, Woldegiorgis et al., 2007, Silva et al., 2008]. The reason might be attributed to the high consumption rate of these drugs. Rather than the amounts sold over the counter, their reported annual prescription in developed countries achieved several hundreds of tons. As an example, 87.5 million prescriptions were written in 2001 for the members of this group only in Germany [Cleuvers, 2004]. Furthermore, the NSAIDs were found to be stable up to 1 year in the environment which increases their risk for accumulation [Khalaf et al., 2009]. Among the NSAIDs, on the other hand, diclofenac has been reported by several authors to be ubiquitously present in most of the surveyed waste, surface, and drinking waters worldwide **[Table 1]**. It is considered one of most important pharmaceutically active contaminants present in the environmental water cycle [Ternes, 2001]. Numerous considerations such as physico-chemical properties, annually consumption rates, environmental occurrence, phototransformation products, biotransformation products, elimination rates, sorption behavior, ecotoxicity data, and the expected environmental hazard shown by risk assessment studies could be the main causes for its importance. Therefore, diclofenac was selected to be under investigation in the present study.

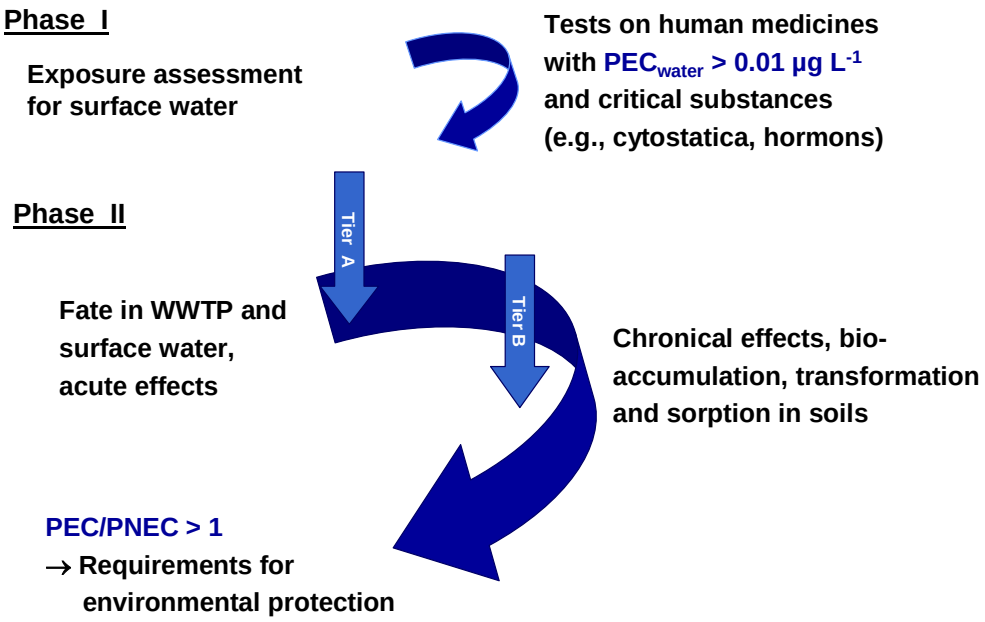


Figure 1: Tiered environmental risk assessment for human pharmaceuticals within the regulatory procedure [EMEA, 2001; Rönnefahrt et al., 2002]

1.1 Environmental relevance of diclofenac

1.1.1 Structure, physico-chemical properties and pharmacological action

Diclofenac is an organic compound containing phenyl, amino and acetic acid moieties. The structure of diclofenac is shown in **Figure 2**.

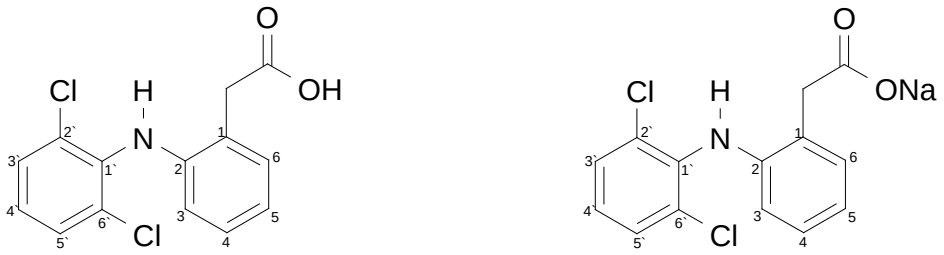


Figure 2: Chemical structure of diclofenac (1a) and diclofenac sodium (1b).

It is known as 2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetic acid (IUPAC name) or acetic acid, (o-(2,6-dichloroanilino)phenyl) [ChemIDPlus]. In the neutral form (molecular formula: $C_{14}H_{11}Cl_2NO_2$, molecular weight: 296.16, and CAS No.:15307-86-5), it has a water solubility of 2.37 mg/L [SCR PhysProp Database, 2010]. In acidic solutions (pH 1.1), the solubility of diclofenac is lower than 1 mg/mL [Palomo et al., 1999, Merck index, 1996]. Due to the presence of carboxylic group and the secondary amino group that can act as proton donor or proton acceptor, it has a Lewis acid–base character [Zilnik et al., 2007]. It is mostly used as the sodium salt which is known as 2-[(2,6 dichlorophenyl)amino]benzoic acid monosodium salt [Merck index, 1996], sodium [o-(2,6-dichloranilino) phenyl] acetate [PharmaDerm], or sodium(O-((2,6-dichlorophenyl)- amino)-phenyl)-acetate [Palomo et al., 1999]. It is a salt of a weak acid with a pKa of 4.16 [Rafols et al., 1997, SCR PhysProp Database 2010] and a distribution coefficient, D_{ow},(n-octanol/aqueous buffer, pH 7.4) of 13.4 [Palomo et al., 1999, Zilnik et al., 2007, Merck index, 1996]. On the other hand, the reported partition coefficient, log P_{ow} or K_{ow}, (n-octanol–water) values of diclofenac are 4.5 [SCR PhysProp Database 2010], 1.9 [Scheytt et al., 2005b], 4.12 [Schmitt-Jansen et al., 2007], and 0.7-4.5 [Johnson et al., 2007]. Its vapour pressure was 6.3×10^{-5} Pa at 25 ºC [SCR PhysProp Database 2010].

Diclofenac sodium is a white to slightly yellow crystalline powder with molecular formula $C_{14}H_{10}Cl_2NNaO_2$, mol wt 318.13, and CAS No. 15307-79-6 [Merck index, 1996]. Its solubility in different solvents is affected by the presence of different heteroatoms (N, O, Cl and Na) which cause high polarizability of the molecule and hence specific interactions with solvents

[Zilnik et al., 2007]. It is freely soluble in methanol > 24 mg/mL, soluble in ethanol, in water (pH 5.2) > 9 mg/mL, in acetone and phosphate buffer (pH 7.2) = 6 mg/mL, in acetonitrile, cyclohexane, and HCl (pH 1.1) < 1 mg/mL, and partially insoluble in ether [Merck index, 1996, PharmaDerm]. At 25 ºC, solubility of diclofenac was found to be 4.45 mg/g in acetone, 1.26 mg/g in ethyl acetate, and 135 mg/g in dimethyl sulfoxide [Zilnik et al., 2007]. Its water solubility varies between 50 g/L (at 25°C, pH 7), 20.4 g/L, and 0.003-21 g/L as published by Perez-Estrada et al. (2005), Zilnik et al. (2007) and Johnson et al. (2007), respectively. The resonating structure and the interaction of diclofenac with water have been described by Iqbal and Chaudhry (2009) as shown in **Figure 3**.

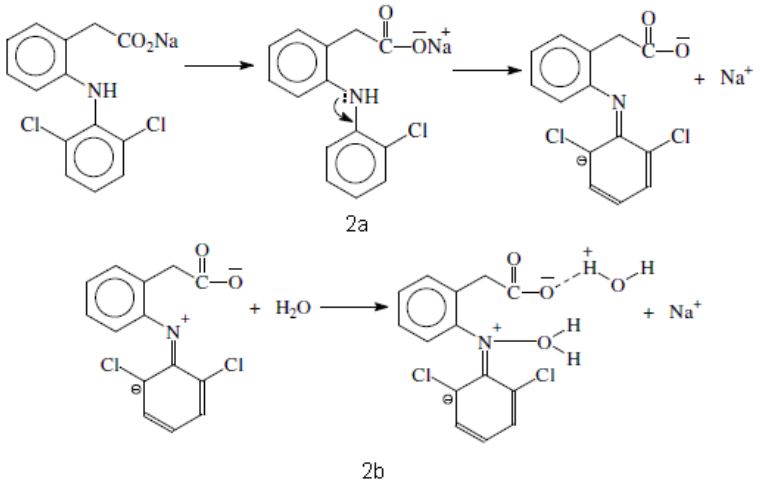


Figure 3: The resonating structure of diclofenac (2a) and the interaction of diclofenac with water (2b)

Pharmacologically, diclofenac can be classified as analgesics, non-narcotic, antirheumatic, cyclooxygenase inhibitors, or NSAIDs [ChemIDPlus]. The latter is the commonly known class for diclofenac. Due to its analgesic, antipyretic, and anti-inflammatory properties, it is mainly used for the treatment of rheumatic diseases (rheumatoid arthritis, osteoarthritis) especially in aged patients and to relieve other pain symptoms such as gout, kidney and gallstones, acute migraines, bursitis, swelling, stiffness, joint, post-operative, post-traumatic and menstrual pain. Inhibition of cyclooxygenase enzyme, which is responsible for the conversion of arachidonic acid to prostaglandin, is the main known mechanism of its action [Pasero et al., 1995, Khalaf et al., 2009]. It is well known and commercially available on the market in various pharmaceutical dosage forms including oral (tablets, capsules), rectal (suppositories), injection (intravenous solutions), ophthalmic (eye drops) and to a large extend dermal dosage forms (ointments and gels). Although the dermal application is the

popular dosage form, 70 % of the worldwide diclofenac sales in 2007 are administrated orally [Zhang et al., 2008].

1.1.2 Consumption rate

The estimation of the consumption rate of diclofenac is very important in the prediction of the environmental occurrence as well as the influence on the ecosystem. The exact consumption rate in any region is very difficult to be calculated since diclofenac can be sold in different ways (over the counter or using prescriptions). Additionally, diclofenac can be found in the markets under different commercial names. In Sweden there are 38 products of diclofenac with different commercial names [Carlsson et al., 2006a]. However, the prescription diclofenac data are available in some countries. In Germany, the annual consumption of diclofenac has been estimated in 1995 at about 75 t/y [Ternes, 1998], in 1999 at 82–250 t/y [BLAC, 2003, Huschek et al., 2004, Scheytt et al., 2005a], in 2001 at 86 t/y [Huschek et al., 2004, Fent et al., 2006]. In 2002 the literature suggested an average diclofenac consumption of 2.5 mg/d person [Ternes, 1998; Heberer, 2002a; Heberer and Feldmann, 2005]. In 2004, diclofenac occupied the second place in the number of prescriptions issued in Germany [Glaeske and Janhsen, 2006, Stuelten et al., 2008]. In England, there were over a million prescription items of diclofenac issued in 1997 [Ashton et al., 2004, Ayscough et al., 2000] and the mount of diclofenac used in 2000 was 26 t/y with an average of 1.4 mg/d person [Jones et al., 2002, Sebastine and Wakeman, 2003]. Other countries such as France, Austria and Australia had reported consumption of diclofenac at 16, 6 and 4 t/y, respectively [Ferrari et al., 2003, Strenn et al., 2004, Khan and Ongerth, 2004].

1.1.3 Metabolization

The most important factor affecting the occurrence of pharmaceuticals in the environment is their pharmacokinetic properties. Pharmacokinetic is mainly established to describe the time period of a drug in the body. After administration of human drugs, they subjected to two main metabolite pathways in the body before they are excreted with urine, feces or both **[Figure 4]**. These pathways are known as phase I and phase II. Phase I metabolism includes oxidation, hydrolysis, reduction, hydration, condensation and isomerization processes while conjugation with sulphate or glucuronic acid is considered as phase II [Merck, 1999]. These transformation processes are very important for pharmaceuticals in order to be more hydrophilic and hence can be easily excreted.

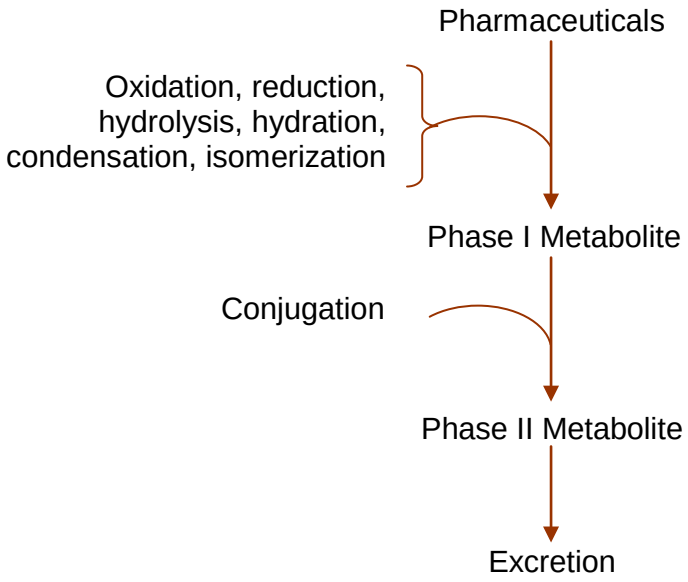
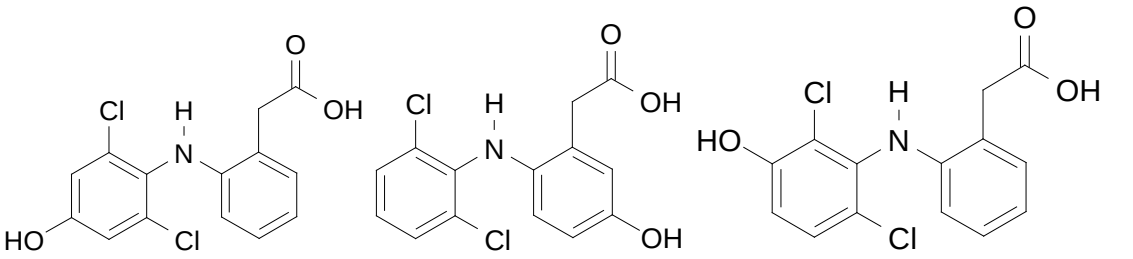


Figure 4: Metabolization of pharmaceutical drugs in humans

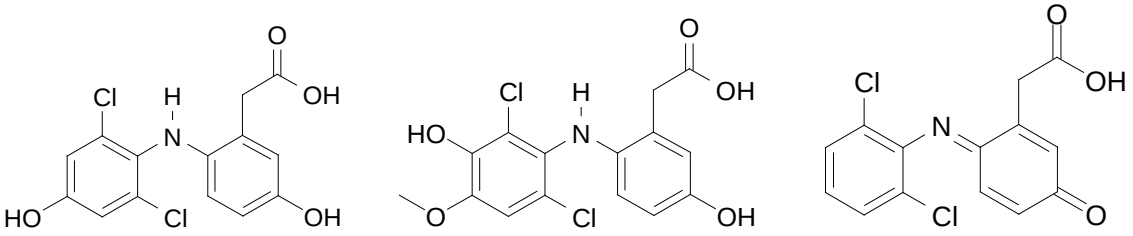
For diclofenac, the predominant process during phase I metabolism is the oxidation which can take place in the phenylacetic acid ring, dichlorophenyl ring or in both resulting in several single or multiple hydroxylated metabolites. These metabolites are 4'-hydroxydiclofenac (16-30 %), 4',5-dihydroxydiclofenac (15 %), 5-hydroxydiclofenac (10 %). 4'-Hydroxydiclofenac and 5-hydroxydiclofenac undergo autoxidation and the highly reactive hepatotoxic p-benzoquinone imines could be then formed. Other minor oxidative metabolites are 3'-hydroxy- (1-2 %) and 3'-hydroxy-4'-methoxy derivatives of diclofenac. Almost 20 % of diclofenac able to pass phase I without oxidation. During phase II metabolism, most of these hydroxylated metabolites and of almost 15 % of diclofenac undergo glucuronide or sulphate conjugations. Conjugations occur either at the carboxyl group of the side chain or at phenolic hydroxyl groups. Approximately 6 % of diclofenac can escape phase II unaltered. Thereafter, all of these compounds are excreted mainly in urine (50-65 % of the oral dose) [Riess et al., 1978, Stierlin et al., 1979, Leemann et al., 1993, Sawchuk et al., 1995, Shen et al., 1999, Belfroid et al., 1999, Schneider and Degen, 1981, Poon et al., 2001, Ashton et al., 2004, Groning et al., 2007, Johnson et al., 2007, Zhang et al., 2008, Perez and Barcelo 2008]. Other authors published highly variable excretion rates (1 to 25 %) for diclofenac as a parent compound [Landsdorp et al., 1990, Ternes, 1998, Mersmann, 2003, Schwaiger et al., 2004, Letzel et al., 2009].

The hydroxylated metabolites were found to be pharmacologically active but 10 to 50 times less potent than diclofenac itself [Menasse et al., 1978, Stierlin et al., 1979, Wiesenberg-

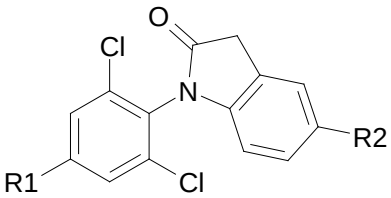
Boettcher et al., 1991, Wishart et al., 2006, Zhang et al., 2008]. Recently, the indolinone structures of 4'-hydroxydiclofenac and 5-hydroxydiclofenac were identified in urine samples as human metabolites of diclofenac [Stuelten et al., 2008b]. The structures of these metabolites are shown in **Figure 5**. In contrast to urinary excretion, very little information is available regarding excretion of diclofenac in feces. Riess et al. (1978) reported 42 % of the oral dose as the maximum amount of diclofenac and its metabolites (free and conjugates) that could be excreted in bile, mainly 10-20 % of 4'-hydroxydiclofenac, 5-10 % of 5-hydroxydiclofenac, and less than 5 % diclofenac. On the other hand, almost 6 % diclofenac is resorbed when applied on the skin and the rest is discharged during washing [Schrey and Wilhelm, 1999, Stuelten et al., 2008a].The half-life of diclofenac in the human body is 1-2 h [Moore et al., 1990. Moore, 1998, Wishart et al., 2006, Zhang et al., 2008].



4'-hydroxydiclofenac 5-hydroxydiclofenac 3'-hydroxydiclofenac



4',5-hydroxydiclofenac 3'-hydroxy-4-methoxy diclofenac p-benzoquinone imine derivative



indolinone derivatives of diclofenac metabolites

Figure 5: Structures of human metabolites of diclofenac

1.1.4 Sources

Understanding of pharmaceutical origins is very necessary for designing effective pollution reduction measures. Pharmaceuticals are able to invade the ecosystem in several ways. In **Figure 6**, the environmental sources of pharmaceuticals are outlined and the main sources are discussed in the following text.

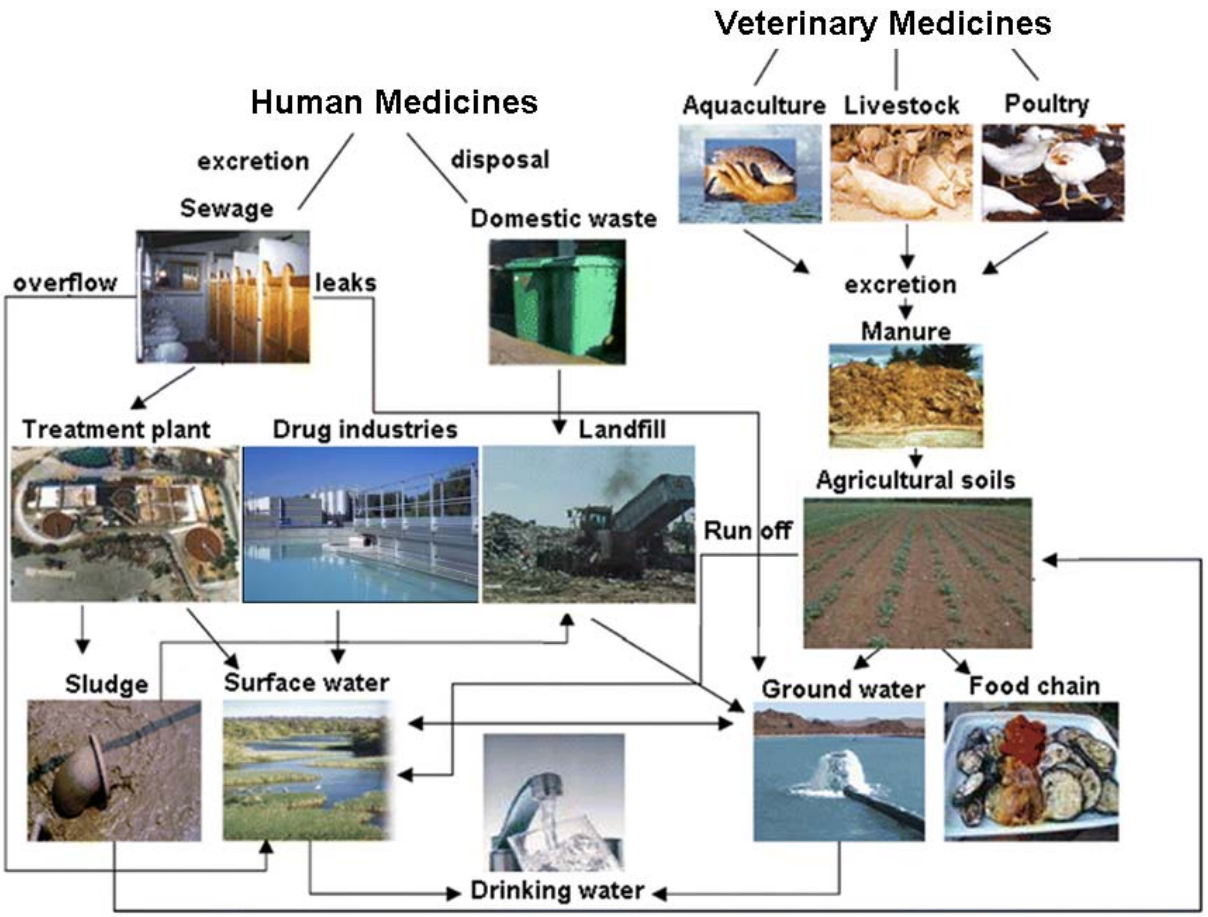


Figure 6: Entry routes and distribution of pharmaceuticals in the environment

Wastewater treatment plants

Wastewater treatment plant (WWTP) effluents are considered the major source for introduction of human pharmaceuticals into the aquatic environment [Bernhard et al., 2006]. They receive pharmaceutical compounds from different places such as homes, hospitals, and cliniks. Disposal of unused or expired pharmaceuticals into toilet is considered to a lesser extends additional source [Kümmerer, 2001]. Once pharmaceuticals, as organic compounds of anthropogenic origin, enter the wastewater treatment process they are either mineralized, deconjugated, degraded, transformed into metabolites or non-extractable residues, sorbed, or pass unaltered through the WWTP effluents to the water bodies [Halling-

Sørensen et al., 1997, 1998, Hartig et al., 1999, Heberer, 2002b, Farre et al., 2001, Kosjek et al., 2005]. Because of their polarity, several drugs are not efficiently removed during wastewater treatment processes and consequently reach surface and even groundwater which are considered the major sources for drinking water [Heberer, 1995, Heberer and Stan, 1997, Heberer et al., 2001, Gros et al., 2006]. Therefore, the elimination performance during the wastewater treatment process is considered the determining factor for presence of pharmaceuticals in the environment [Alcock et al., 1999, McArdell et al., 2003, Ternes, 1998, Ternes et al., 1999].

After reaching the water bodies, pharmaceuticals can undergo further processes such as biotransformation, phototransformation and/or sorption onto the sediment. It could be additionally reach groundwater through bank filtration. Since small amounts of the total oral dose are excreted in free form, diclofenac (as a parent compound) is expected to be generated by means of different processes. The cleavage of the conjugated form of diclofenac by β-glucuronidase enzymes seems to be one of the main sources of regeneration of diclofenac in WWTP [Ternes, 1998, Belfroid et al., 1999, Hirsch et al., 1999, Ternes et al., 1999, Ashton et al., 2004, Schwaiger et al., 2004, Vieno et al., 2007]. This process might be responsible for the negative removal results reported for diclofenac [Lishman et al., 2006, Sebok et al., 2008]. Another possible source for the formation of diclofenac is the ester hydrolysis of aceclofenac. In batch reactors, biodegradation experiments using mixed liquor from the conventional activated sludge aeration tank showed that 90 % of the initially applied aceclofinac amount is converted to diclofenac [Perez and Barcelo, 2008]. Dermal applications are, additionally, considered other major source for diclofenac in the wastewater, since small amounts (10 %) are only absorbed through the skin and the remaining amounts (90 %) are discharged directly after washing [Heberer and Feldmann, 2005].

Pharmaceutical industries

As a result of the good manufacturing practice regulations, the wastewater treatment plants of pharmaceutical industry in the developed countries are expected to provide high elimination efficiency for most of the wastewater constituents including diclofenac. Therefore, pharmaceutical manufactures were excluded for long time as a major emission source for pharmaceuticals in Europe and USA [Schroeder, 1999, Sacher et al., 2001]. Recently, in New York, pharmaceutical formulation facilities have been reported to release drugs into the environment at concentration levels ranging from 10 to 1000 times higher than those typically found in WWTP effluents [Phillips et al., 2010]. In the developing countries, several companies do not treat its effluents before discharging into rivers. In Pakistan,

pharmaceutical industrial waste effluents are considered one of the main sources of diclofenac in River Malir [Scheurell et al., 2009]. In Slovenia, the discharge effluent of the Novo Mesto pharmaceutical factory is expected to be one the major sources of diclofenac in River Krka [Antonic and Heath, 2007]. In Taiwan, diclofenac has been detected in wastewater effluents of drug production facilities in a median concentration of 20 µg/L [Lin et al., 2008]. Not only diclofenac but also other drugs have been detected at high concentration levels due to drug manufactures. As an example, ciprofloxacin was detected in the effluent of water treatment plant, receiving wastewater from about 90 bulk drug manufacturers in Patancheru, India (a major production site of generic drugs for the world market), at a concentration (up to 31 mg/L) exceeds the toxic levels to some bacteria by over 1000-fold [Larsson et al., 2007]. Therefore, pharmaceutical industrial effluents are important local point sources of drug residues in the environment [Kosjek et al., 2007a, Zuccato et al., 2000]. Furthermore, landfills receiving pharmaceutical industrial waste and have no leachate collection systems are considered one of the emission sources of drugs into the surrounding aquifers. For example, in Denmark several pharmaceuticals were indentified in the ground water located adjacent to landfill containing 85 000 t of drug industrial waste [Holm et al., 1995]. In a similar way, diclofenac can leach from landfills through the subsoil and from contaminated surface water through bank filtration into the ground water [Heberer et al., 1998].

Fertilizers, water for irrigation and aquaculture

When used as fertilizers in agriculture, WWTP effluents, sewage sludge, solid and liquid manure may result in transport of human or veterinary pharmaceuticals into the surface waters via agricultural runoff [Hirsch et al., 1999, Lindsey et al., 2001, Sacher et al., 2001, Campagnolo et al., 2002, Pedersen et al., 2003]. Diclofenac, as a human and veterinary drug, has a potential to enter the food chain and reach drinking water especially when contaminated or untreated wastewater used in crop irrigation [Munoz et al., 2009, Siemens et al., 2008]. Although the analysis of runoff samples, from fields irrigated with treated effluent, indicated presence of low levels of diclofenac, other pharmaceuticals such as carbamazepine, gemfibrozil, carisoprodol have been detected in a concentration up to 2 µg/L [Pedersen et al., 2005]. This finding showed that fertilizers should not be neglected as a source for pharmaceutical contaminants. Additionally, aquaculture fish farms play a significant role in the presence of drugs in the environment [Calamari et al., 2003, Zuccato et al., 2006].

1.1.5 Occurrence

The presence of pharmaceuticals in the environment is closely related to their fate. After excretion, the ideal fate of drug residues should be mineralization by the microorganisms to carbon dioxide and water. Unfortunately, most of medicinal products do not undego the ideal fate. Therefore, they could be detected in the aquatic environment. Own to several aspects such as high consumption rate and polarity, diclofenac has been detected in almost all surveys of sewage, surface, ground, and drinking water samples at different concentration levels ranging from ng/L to µg/L. In Germany, Stumpf et al. (1996) were the first authors all over the world who detected diclofenac in sewage and river water. The next was Ternes (1998) who detected diclofenac in 49 WWTP effluent samples and 22 river samples at 90-percentile concentration of 1.6 and 0.8 µg/L, respectively. Afterwards, Heberer et al. (1998) investigated waters of Berlin in a screening study. They pointed to diclofenac as the most second drug (after clofibric acid) occurring in their collected samples. Although diclofenac showed 100 % elimination during bank filtration study [Schmidt et al., 2003], it has been detected in ground, drinking and even tap water. It was detected by Heberer et al. (1998) for the first time in groundwater of a drinking water plant at concentration level of 0.38 µg/L and then by Sacher et al. (2001) at concentration of 0.6 µg/L. Furthermore, samples taken from a private drinking water tap in Berlin showed presence of diclofenac at trace level concentration of less than 0.01 µg/L [Heberer, 2002b]. In the Mediterranean region, diclofenac was found in six of seven drinking water wells at concentrations around 2 ng/L [Rabiet et al., 2006]. Furthermore, Cleuvers (2004) detected diclofenac in drinking water at maximum concentration of 6 ng/L. The highest concentrations of diclofenac, up to 15 and 30 µg/L, were detected in German surface water and Taiwanese wastewater effluents by Jux et al. (2002) and Lin et al. (2008), respectively. An average concentration of diclofenac of 0.15 µg/L was estimated in German river water [Poseidon, 2006]. From different WWTP, the specific average emission of diclofenac for each person was estimated in the range of 0.28-0.60 mg/d in Germany [Letzel et al., 2009, Heberer and Feldmann, 2005], 0.26 to 0.67 mg/d in Switzerland [Buser et al., 1998b, Tixier et al., 2003, Tauxe-Wuersch et al., 2005], and at 0.088 mg/d in Canada [Lishman et al., 2006]. Some of other data regarding environmental occurrence of diclofenac are listed in **Table 1**.

Table 1: Average concentrations (µg/L) of diclofenac in the environment

WWI	WWE	SW	DW	MA	References
---	< 4.5	0.5	0.06	GC/MS	Stumpf et al. (1996) Germany
2.1	---	1.2	---	GC/MS	Ternes (1998) Germany
---	---	1	nd-0.38	GC/MS	Heberer et al. (1998) Germany
---	---	0.30-0.42	---	GC/MS	Sacher et al. (1998) Germany
0.5-1.9	0.31-0.93	<0.001-0.37	---	GC/MS	Buser et al. (1998b) Switzerland
0.8	0.72-0.2	0.01-0.10	---	GC/MS	Stumpf et al. (1999) Brazil
0.8-3.2	---	1	---	GC/MS	Werres et al. (2000) Germany
0.1–0.7	---	n.d-0.15	---	GC/MS	Oellers et al. (2001) Germany
---	---	---	0.6	GC/MS	Sacher et al. (2001) Germany
---	---	a) 0.02-0.04 b) 0.03-0.39	---	a) LC/MS b) CE/MS	Ahrer et al. (2001) Austria
---	nd-0.4	nd-0.6	---	LC/MS/MS	Farre et al. (2001) Spain
---	0.1-0.7	nd-0.15	---	GC/MS	Oellers et al. (2001) Switzerland
---	---	---	<0.01	CE/MS	Heberer (2002b) Germany
---	---	0.01-0.03	0.005-0.04	GC/MS	Heberer (2004) Germany
---	---	15	---	GC/MS	Jux et al. (2002) Germany
---	0.99	---	---	GC-MS	Tixier et al. (2003) Switzerland
a) 1.9-8.4 b) 2-2.4	a)1.1-2.7 b)0.1-0.3	a) 0.01 -0.02 b) 0.002–0.006	nd	a) ELISA b) GC/MS	Deng et al. (2003) Austria and Germany
---	0.08	---	nd	GC/MS	Drewes et al. (2003) USA
---	0.35-0.46	0.021-0.09	---	LC/MS/MS ESI	Hilton et al. (2003) UK
---	nd-0.06	nd-0.5	---	LC/MS/MS ESI	Marchese et al.(2003) Italy
0.07	0.07	---	---	GC/MS	Lee et al. (2003) Canada
---	---	0.01-0.05	---	GC/MS	Wiegel et al. (2004) Germany
---	<0.02-2.3	<0.02-0.5	---	LC/MS/MS	Ashton et al. (2004) United Kingdom

Table 1: Continued

WWI	WWE	SW	DW	MA	References
0.16	0.12	0.01-0.12	---	GC/MS	Bendz et al. (2005) Sweden
0.35	---	0.002-0.04	---	LC/MS/MS ESI	Lindqvist et al. (2005) Finland
---	---	0.01–0.3	nd	GC/MS	Kosjek et al. (2005) Slovenia
0.021-0.148	0.032-1.420	0.03-0.07	nd	LC/MS/MS ESI	Hernando et al.(2006) Spain, Belgium, Germany, Slovenia
---	0.06–1.9	---	---	LC/MS/MS	Gomez et al. (2006) Spain
---	0.2-0.5	0.001-0.03	nd-0.003	GC/MS	Rabiet et al. (2006) France
0.9-1	0.26- 0.6	>0.008	---	LC/MS/MS ESI	Roberts and Thomas, (2006), UK
1.6-4.4	0.65-1.3	---	---	LC/MS	Bernhard et al. (2006) Germany
----	----	nd-0.089	---	CE/MS	Antonic and Heath (2007), Slovenia
0.006-5.9	---	---	---	LC/MS/MS, TOF/MS/ ESI	Bueno et al. (2007) Spain
0.2–3.6	0.14–2.2	---	---	GC/MS	Gomez et al. (2007) Spain
---	---	0.25	---	GC/MS	Moeder et al. (2007) Germany
---	nd-0.5	nd- 0.04	---	LC/MS/ ESI	pedrouzo et al. (2007) Spain
13-17	1-7	---	---	LC/MS/MS	Radjenovic et al. (2007), Spain
---	1-5.1	---	---	LC/MS/MS ESI	Stuelten et al. (2008b) Germany
0.05-4.2	---	---	---	GC/MS and LC/MS	Terzic et al. (2008) Balkan Region
---	<0.002-0.03	0.02-0.06	---	LC/MS/MS ESI	Chen et al. (2008) Taiwan
0.002-0.17	---	0.004-1	---	GC/MS	Togola and Budzinski (2007), France
---	0.21–0.48	0.001–0.03	nd–0.002	GC/MS	Togola and Budzinski (2008), France
---	0.01-30	---	---	LC/MS/MS ESI	Lin et al. (2008) Taiwan
2.6	3.2	---	---	GC/MS/MS	Sebok et al. (2008) Hungary
---	0.12-2.2	0.03-0.14	---	LC/MS/MS ESI	Letzel et al. (2009) Germany
---	---	0.1-8.5	---	GC/MS	Scheurell et al. (2009) Pakistan

Table 1: Continued

WWI	WWE	SW	DW	MA	References
---	---	0.006-0.02	---	LC/MS/MS ESI	Khalaf et al. (2009) Sweden
---	0.006-1.3	---	---	LC/MS/MS	Munoz et al. (2009) Spain
0.35	0.01	---	---	LC/MS/MS ESI	Perez and Barcelo (2008), Spain

nd= not detected, --- = not investigated, WWI= wastewater influent, WWE= wastewater effluent, SW= surface water, DW= drinking water, MA= method of analysis

1.1.6 Elimination

The elimination processes of pharmaceutical compounds mainly occur in the WWTP following different mechanisms. Biotransformation is considered the most important elimination process in which microorganisms are used to mineralize the pharmaceuticals to water and carbon dioxide, or degrade them to non-effective forms. Sorption to activated sludge is another possible method for elimination. Because the wastewater treatment process is performed in the open air, wastewaters are almost exposed to the sun light. Hence, some pharmaceuticals can undergo phototransformation. Some non polar substances, alternatively, can be removed by volatilization. Therefore, the expected elimination mechanisms of diclofenac, either in the WWTP or in surface water, are biotransformation, phototransformation or to some extend sorption. The state of art showed that extensive field and laboratory elimination experiments have been conducted to investigate the removal efficiency of diclofenac in conventional activated sludge, membrane bioreactor reactors, column experiments and in surface water. In field studies, the removal efficiency of diclofenac is usually calculated as the concentration differences between its influent and effluent levels.

Sewage

The elimination of diclofenac during sewage treatment seems to be rather complicated. No clear results and high data deviation have been obtained for its removal efficiencies. The results from the EU project Poseidon [Poseidon, 2006] showed that the literature data on its elimination is very contradictory.

Following primary sedimentation, aeration and phosphate removal treatment, Ternes (1998) reported 69 % reduction in diclofenac concentration. Comparing influent and effluent concentrations, Lee et al. (2003) did not report diclofenac removal rates, Poiger et al. (2001)

observed removal ranging from 5 % to 50 % while 0 % to 74 % has been reported by Clara et al. (2005a). An adapted lotic biofilms (*Cytophaga-Flavobacterium* and *γ-Proteobacteria* bacterial groups) grown in presence of diclofenac (100 µg/L) showed degradation ranging from 10 to 25 % of the initial concentration within 4 days [Paje et al., 2002]. During anaerobic digestion of sewage sludge, 69 % removal rates of diclofenac have been reported by Carballa et al. (2007). In batch reactors experiments, 50 % of diclofenac was reported to be degraded within 6 days in mixed liquor from conventional activated sludge aeration tank [Perez and Barcelo, 2008]. The other published removal data were 9-75 % [Stumpf et al., 1999], 22 % [Bendz et al., 2005], 20-40 % [Joss et al., 2005], 9-60 % [Lindqvist et al., 2005], 70 % [Roberts and Thomas, 2006], 60 % [Gomez et al., 2007], 40-80 % [Kimura et al., 2007], 49-59 % [Kosjek et al., 2007a], and 100 % [Thomas and Foster, 2004, 2005].

On the other hand, several groups described diclofenac as a persistent pharmaceutical compound even upon biological treatment. For example, the incubation of water influent with activated sludge under aerobic conditions showed no degradation of diclofenac [Buser et al., 1998b]. Very poor elimination values (1-6 %) for diclofenac was reported in a pilot WWTP by Zwiener et al. (2001). In a 55-h biodegradation experiment, diclofenac was reduced to 4 % at maximum even when oxic biofilm reactors was used [Zwiener and Frimmel, 2003]. In a biodegradation test, concentrations of 20 mg/L diclofenac were shown to be remained unaffected by the presence of activated sludge over a period of 28 days [Quintana et al., 2005]. In a biological filter plant study, diclofenac exhibited removal of only 5-9 % [Johnson et al., 2007]. Joss et al. (2006) considered diclofenac as a non-biodegradable pharmaceutical compound showing elimination constant $K_{biol} < 0.1$ L/g_{ss} d while in the EU project Poseidon [Poseidon, 2006] K_{biol} at 0.25 ± 0.2 L/g_{ss} d and removal ranging from 15 to 40 % were estimated in the aerated compartment of nutrient removing plants (plants in which the removal of nitrogen and/or phosphorous from wastewater are performed using biological methods of treatment in order to improve the wastewater quality). Other authors reported elimination of diclofenac less than 20 % either in batch or field experiments are Moehle et al. (1999), Heberer et al. (2002), Strenn et al. (2004), Clara et al. (2005b), Tauxe-Wuersch et al. (2005), Knopp et al. (2007), Kimura et al. (2007).

This variation could be attributed to several factors such as equipment used, treatment steps, temperature, weather, solid retention time, microbial activity of the sludge, type(s) of microorganisms existed in the sludge, sorption characteristics of the sludge, and toxic effects of the industrial wastewater entering the plants that also affect the microbial activity [Ternes, 1998, Paje et al., 2002, Tixier et al., 2003, Lindqvist et al., 2005, Kimura et al., 2007, Letzel

et al., 2009]. Coagulation-flocculation resulting from the action of ferric chloride and aluminium sulphate, cations which are frequently added to the biological reactor in order to precipitate phosphorus [Suarez et al., 2008], may enhance the binding of diclofenac to the suspended solids throughout the trivalent cations [Carballa et al., 2005]. Thus, removal of diclofenac by means of such reaction could be another explanation of the discrepancies concerning its removal efficiencies reported in the literature. However, Ternes et al. (2002) reported no considerable elimination of diclofenac, in lab-scale and waterworks, from row water after treatment with ferric chloride. In contrast, high elimination (> 90 %) of diclofenac was reported after filtration through activated carbon [Ternes et al., 2002, Westerhoff et al., 2005, Vieno et al., 2007].

Addtitionally, it was found that application of membrane bioreactor reactors usually shows higher elimination efficiency for diclofenac than conventional activated sludge. Using anoxic biofilm reactor, Zwiener and Frimmel (2003) reported an elimination rate of diclofenac up to 40 %. In a laboratory scale, application of membrane bioreactor reactors resulted in 78-87 % removal of diclofenac while application of conventional activated sludge resulted in only 50-60 % removal [Bernhard et al., 2006, Radjenovic et al., 2007]. Samples collected from WWTP showed no diclofenac removal (3 %) during conventional activated sludge treatment while 85 % of the initially present concentration was eliminated following passage through the membrane bioreactor reactors [Perez and Barcelo 2008]. In contrast, other studies reported no [De Wever et al., 2007] or partial removal [Clara et al., 2005b] of diclofenac after application of those treatments. Another study proved that the low frequency ultrasound irradiation, acidic conditions and presence of dissolved air increased the elimination of diclofenac in wastewater [Naddeo et al., 2009].

The most interesting aspect concerning diclofenac is the phenomenon of increasing effluent than influent concentrations during the same study. Lishman et al. (2006) reported negative removal rates up to -143 % in several samples (12 of 26). In Hungary, the concentration of diclofenac was found to be 2.6 µg/L and 3.2 µg/L in influent and effluent, respectively [Sebok et al., 2008]. Other negative removal rates were observed by De Wever et al. (2007) and Perez and Barcelo (2008). Several others attributed the increased effluent concentrations to the cleavage of glucuronide conjugates by enzymatic processes (β-glucuronidase enzymes) in the treatment plants [Ternes, 1998, Miao et al., 2002, Roberts and Thomas, 2006, Lishman et al., 2006, Vieno et al., 2007, De Wever et al., 2007, Sebok et al., 2008, Perez and Barcelo, 2008]. Such a phenomenon renders the evaluation of biological treatment processes very difficult, since exact biodegradation data could not be obtained. Another

reason could be the daily concentration fluctuations during the sampling time [Clara et al., 2004]. Roberts and Thomas (2006) attributed this phenomenon to the suppression of the MS/MS detector signal due to the higher concentration of organic matter in the raw wastewater influent than in the treated effluent.

Surface and pure water

Diclofenac showed minimal chemical and biological degradation in the lake Greifensee water when incubated in a glass vessel in the dark while rapid elimination by meanes of photodegradation was observed [Buser et al., 1998b]. They reported half-life time less than 1 h after exposure of diclofenac to sunlight. The same behavior was observed by Packer et al. (2003). They reported, in both purified and Mississippi River water, half-life time of 39 min after exposure of diclofenac to the sunlight in August. They additionally expected increasing of the half-life time to approximately 3-8 h in winter due to decrease of the sunlight light intensity [Packer et al., 2003]. In purified water, Andreozzi et al. (2003) reported 5 d as a half-life of diclofenac during the spring to summer time while, in the midsummer time, Schmitt-Jansen et al. (2007) reported 3.3-6.4 h.

As well as laboratory experiments, efficient removal again due to photodegradation was observed for diclofenac in the aquatic environment. Buser et al. (1998b) reported that 90 % of the diclofenac entering a major tributary of Swiss Lake Greifensee is significantly eliminated by photolytic degradation and neither sorption nor accumulation could be found in the sediments of the lake. Under the environmental condition, diclofenac showed short half-life time ranging 4 to < 24 h [Buser et al., 1998b, Ashton et al., 2004]. However, longer half-life (8 days) in the Lake Greifensee over a summer period (August-October) was reported by Tixier et al. (2003). The metabolic rate in humans could influence the half-life time of diclofenac in the aquatic environment. Bendz et al. (2005) proposed that substances which have high metabolic rate in humans (low excretion rate as parent compound) exhibit short half-lifes in the aquatic environment almost less than 1-2 days and vice versa. Therefore, Letzel et al. (2009) were not able to detect diclofenac in almost all river samples collected 10 km away from the WWTP discharge site. However they assumed that locally, directly behind WWTP discharge points, higher amounts could occur in surface water. Furthermore, diclofenac has been reported to be susceptible not only to light but also to ozonation where extensive elimination (90-97 %) from pure water samples can be realized at ozone doses of 0.5 mg/L [Zwiener and Frimmel, 2000, Ternes et al., 2002, Vieno et al., 2007].

Soil and sediment

In different types of soil, the fate of ^{14}C-diclofenac was investigated revealing higher biodegradability in clayey silt soil than silty sand soil where 23 and 43 %, respectively, of the initially applied radioactivities could be extracted after 3-d incubation interval [Kreuzig et al., 2003]. In sandy sediment, a high persistence of diclofenac to biodegradation was suggested by Ternes et al. (2002) during investigation of diclofenac for 28 days under aerobic and anaerobic conditions, while Groning et al. (2007) reported rapid removal of diclofenac (93 % within 5 days) under aerobic conditions. In a biodegradation experiment, the effect of water-sediment interaction on the elimination of diclofenac was investigated by Kunkel and Radke (2008) in bench scale channel applying water from the River Roter Main at different velocity. They observed that the fast influx of water, which maintain adequate transfer of solutes and oxygen to the sediment, results in rapid removal of diclofenac (DT_{50} 3.2 d) while the slow influx result in slow removal rates (DT_{50} of 8.5 d). In contrast, they reported that diclofenac could not be eliminated within 12 days when fortified in only river water (without sediment) assuming that sorption to suspended matter, microbial degradation, and hydrolysis of diclofenac are excluded in river water.

Sorption and Column studies

Sorption is considered one of the key factors controlling the input, transport, and transformation of chemicals in the environment. Depending on their lipophilicity, there are mainly two coefficients used for assessment of sorption of organic compounds based on the octanol/water partitioning coefficient (K_{ow}) and on the normalized organic carbon-based coefficient (K_{oc}). Since most of pharmaceuticals often contain polar functional groups (e.g. carboxylic, aldehyde and/or amine), these coefficients can be used only as an approximate guide to help in the interpretation of their sorption behavior. However, studying sorption behavior of pharmaceuticals, especially in sediments, is very important in predicting the mobility and leaching tendencies of these compounds at the bank filtration sites. Additionally, the sorption coefficient K_d values can be used for estimation of the particle associated fraction of pharmaceuticals in surface water and hence calculation of their freely dissolved fractions [Stein et al., 2007, Ramil et al., 2010]. Medicinally, it is observed that the drugs abuse has increased considerably. Therefore, sorption of pharmaceuticals onto solids, other than sediments, such as activated charcoal, magnesium silicate, magnesium stearate, or talc powder would be very helpful in treatment of acute toxicity resulting from drug overdose especially in case of drugs that do not have specific antidote [Tella and Owalude, 2007].

Rather than lipophilicity, physico-chemical properties of the solids, humification stage of the solid organic matter (SOM), the level of clay and organo-clay complexes, many factors can affect the interaction of pharmaceuticals with special parts of organic matter or with minerals such as temperature, pH, ionic strength, cation bridging, cation exchange and hydrogen bonding [Goss and Schwarzenbach, 2001, Golet et al., 2003, Ternes et al., 2004, Chefetz et al., 2008, Carballa et al., 2008].

For diclofenac, no considerable sorption (based on the relative concentration C/C_o) was observed in batch experiments using sand (taken from the underground of a groundwater catchment area) under natural aerobic and anoxic conditions [Ternes et al., 2002]. Additionally, the sorption behavior (depending on the K_d values) onto different soil types was studied by Kreuzig et al. (2003). They expected leaching potential of diclofenac through sandy soil where K_d was estimated at 4 L/kg. However, leaching experiment using two soil types with different organic carbon and clay contents showed strong adsorption of diclofenac as it could not be detected in the leachate [Drillia et al., 2003]. The published sorption coefficient (K_{oc}) of diclofenac was found to be 830 L/kg [Antonic and Heath, 2007]. Additionally, K_d of 9 L/kg was reported by Barron et al. (2009) for diclofenac in agriculture soil. In more detail, the effect of organic matter on the mobility (column experiments) and sorption/desorption (batch experiments) behavior of diclofenac in different sterilized soil layers (with different solid organic matter contents) using tap water or secondary-treated wastewater (STWW) was studied by Chefetz et al. (2008). Upon their column experiments, they classified diclofenac as a slow mobile compound in high SOM containing soil while its mobility increases significantly in SOM poor soils. Additionally, they reported lower sorption of diclofenac onto the soil layers in case of using STWW (32 %) than tap water (64 %). They attributed these results to the formation of diclofenac complexes with the dissolved organic matter (DOM) or with organic and inorganic suspended materials which were found to be higher in the STWW solution. In contrast, in their batch experiments using soil with high SOM contents, diclofenac exhibited lower sorption in case of tap water (K_F= 8.81) than STWW (K_F=11.90). They assumed that co-sorption and/or cumulative sorption of diclofenac with the dissolved organic matter (DOM) to the soil's solid matrix (mainly SOM) are the suitable explanation of these results. Opposite results were obtained when soil with low SOM was used (higher sorption in case of tap water than STWW). They explained these results by the competitive sorption between diclofenac and DOM on the few SOM sites and hence decreased the sorption affinity of diclofenac. Margon et al. (2009) attributed the transport of diclofenac through the coarse soil profile to the formation of diclofenac/humic acids micelles as a result of the interaction between diclofenac and the humic acids. In another laboratory

study, diclofenac showed negligible sorption onto sediment particles while limited sorption may occur at lower pH [Buser et al., 1998b]. The sorption coefficient of diclofenac was found to be quite low and the distribution coefficient (K_d values) was estimated between 0.55 to 4.66 in sandy sediment [Scheytt et al., 2005a] and at 4.2 in river sediment [Krascsenits et al., 2008]. Furthermore, the water/sludge distribution coefficient of diclofenac was found to be 16, 66, and 105 L/kg as reported by Ternes et al., 2004, Carballa et al., 2008, and Barron et al., 2009, respectively, indicating that sorption tendency to sludge was relatively low. Similar behavior was reported by Joss et al. (2005), Bernhard et al. (2006) and Knopp et al. (2007) during investigation of diclofenac in presence of sludge. On the other hand, Bui and Choi (2009) reported high adsorption rate (88 %) for diclofenac on the synthesized mesoporous silica SBA-15.

Additionally, column experiments with groundwater model systems indicated a moderate persistence of diclofenac. The elimination was between 60 % and 80 % in aerobic systems and between 40 % and 60 % in anaerobic systems [Preuss et al., 2001]. Other column experiments performed by Scheytt et al. (2004) and Mersmann et al. (2002) showed high retention factors of 2.0 and 2.6, respectively. Furthermore, sorption of 35 % [Scheytt et al., 2006a] and 79 % [Scheytt et al., 2006b] was reported for diclofenac probably due to degradation under the experimental conditions. These results may indicate that diclofenac has low or no mobility at bank filtration sites. In Germany, complete elimination (100 %) was observed for diclofenac during a bank filtration study [Schmidt et al., 2003].

1.1.7 Phototransformation

Phototransformation may be caused by solar radiation. The most dangerous part of the solar radiation is the highest energetic of the UV region. Naturally, UV radiation passing the upper atmosphere has frequencies of 200-400 nm. In the upper atmosphere, the most energetic part of the UV radiation (200-290) is absorbed, almost by nitrogen, oxygen, carbon dioxide and ozone. Approximately, 10 % of the UV is able to reach the earth's surface in the range of 290 - 320 nm. Artificially, the sunlight can be simulated using radiation sources such as Xe-lamp, mercury arc lamps and germicidal lamps that are able to emit spectrum starting from 290 nm [Jagger, 1985, Moore, 2002, Tixier et al., 2003, Meite and Mazellier, 2006, Mendez-Arriaga et al., 2008]. Generally, substances with absorption spectra that have a good overlap with the radiation source spectrum are considered photo-reactive compounds. The published absorption maxima of diclofenac are 285 [Moore et al., 1990, Zilnik et al., 2007, Cavalheiro et al., 2008], 286 [Chefetz et al., 2008], 275 [Moore, 2002], 273 in aqueous acid and 275 nm in aqueous alkali [El Haj et el., 1999]. However, the absorption band which runs to 325 nm

(allowing complete overlap on the emission spectrum above 290 nm) is responsible for the well known photosensitivity phenomenon of diclofenac **[Figure 7].**

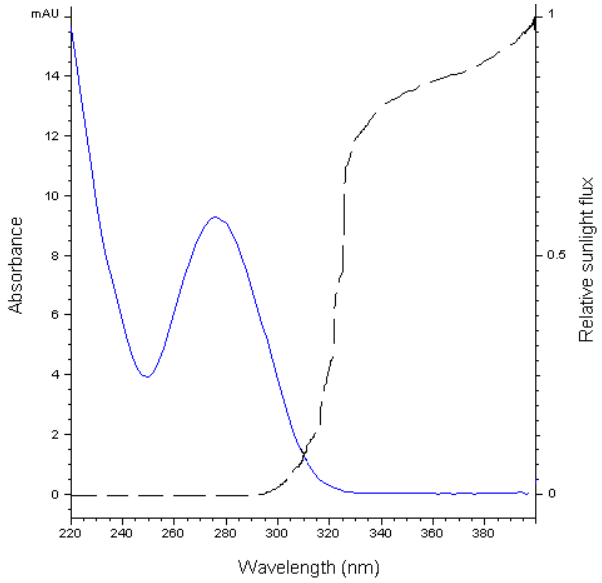


Figure 7: UV spectrum (λ = 220-400 nm) of diclofenac (—) against sunlight spectrum (---) on the earth's surface

This phenomenon has been reported by several authors in different laboratory studies [Moore et al., 1990, Encinas et al., 1998b, Ravina et al., 2002, Andreozzi et al., 2003, Gaudiano et al., 2003, Agueera et al., 2005, Perez-Estrada et al., 2005, Schmitt-Jansen et al., 2007, Kim and Tanaka, 2009] as well as under environmental conditions [Buser et al., 1998b, Poiger et al., 2001, Tixier et al., 2003, Packer et al.,2003, Bartels and von Tuempling Jr, 2007, Scheurell et al., 2009]. All of them observed a fast phototransformation either in surface and/or in pure waters when diclofenac is exposed to light.

In purified artificially fresh and river waters, direct photolysis (under natural sunlight) and/or indirect photolysis using Fenton reagent as a source of (•OH) radicals or isopropanol as hydroxyl radical scavenger were frequently studied. After exposure to sunlight for almost 30 h, Agueera et al. (2005) and Schmitt-Jansen et al. (2007) found that more than 70 and 90 %, respectively, of the initially applied diclofenac was degraded. Another study reported up to 75 % decrease of diclofenac concentration within 2 h of exposure to Xe lamp [Mendez-Arriaga et al., 2008]. In presence of photo-Fenton reagent, complete transformation of diclofenac was observed in a time period ranging from few minutes [Ravina et al., 2002] to 60 min [Perez-Estrada et al., 2005] and totally mineralized after 50 min of exposure to a lamp emitting light at 254 nm [Ravina et al., 2002] or after 100 min of exposure to sunlight [Perez-Estrada et al., 2005]. Packer et al. (2003) reported more rapid transformation for diclofenac

in presence of isopropanol. It has to be noticed that, the photolysis of diclofenac is accompained by decrease in the pH value (ranging from 6.5 to 3) due to elimination of HCl [Perez-Estrada et al., 2005, Mendez-Arriaga et al., 2008].

Depending on the treatment applied, different phototransformation products of diclofenac such as hydroxylated intermediates, C-N cleavage products [Vogna et al., 2004], quinone imine derivatives [Perez-Estrada et al., 2005] and carbazole derivatives have been found **[Table 2]**. Carbazole derivatives (especially 8-chlorocarbazole-1-acetic acid) are considered the most frequently detectable compounds formed by photolysis of diclofenac [Moore et al., 1990, Encinas et al., 1998b, Buser et al., 1998b, Poiger et al., 2001, Agueera et al., 2005, Cavalheiro et al., 2008, Scheurell et al., 2009]. It was isolated (using reversed phase column chromatograpy) and identified by LC/MS, GC/MS (after derivatization with diazomethane), high resolution EI/MS and NMR [Moore et al., 1990] and by electron impact MS and NMR [Encinas et al., 1998b]. In comparison to diclofenac, it was characterized as more polar [Moore et al., 1990], photodegradable [Moore et al., 1990, Encinas et al., 1998b] and more photosensitive [Poiger et al., 2001]. However, due to the lack of authentic reference chemicals, the photochemistry of diclofenac and its phototransformation products were studied by Encinas et al. (1998a) using 2,6-dichlorodiphenylamine and 1-chloro-9H-carbazole as related model compounds. The nature of the reaction including conversion of diphenylamine to carbazole, in presence of light, has been already published since 1963 [Bowen and Eland, 1963]. This reaction has been reported for the first time for pharmaceuticals when carbazole derivatives were obtained by irradiation of meclofenamic acid using visible or UV light [Philip and Szulczewski, 1973].

On the other hand, the artifact [1-(2,6-dichlorophenyl) indolin-2-one] was reported as one of the major phototransformation products of diclofenac in pure water [Bartels and von Tuempling Jr., 2007] and recently in surface water [Scheurell et al., 2009]. Using of GC/MS in their sample analysis could be the reason for their observation, since the formation of artifact at high temperature may be possible. Nevertheless, phototransformation appears to be the most important natural elimination process for diclofenac in the aquatic environment at all. Therefore, several authors applied UV radiation in removal of diclofenac from polluted and drinking water especially in combination with of photo-Fenton, photocatalytic titanium dioxide, hydrogen peroxide, and/or ozone. These combinations, which are known as the advanced oxidation process, were found to be the best method achieving almost complete mineralization of diclofenac [Huber et al., 2003, Vogna et al., 2004, Mendez-Arriaga et al.,

2008, Benotti et al., 2009, Felis et al., 2009, Felis and Miksch, 2009, Garcia-Araya et al., 2010].

Table 2: Selected transformation products of diclofenac mainly due to photolysis processes

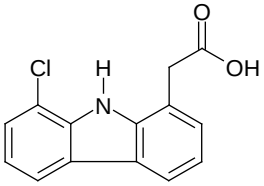
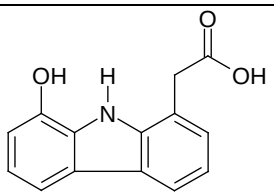
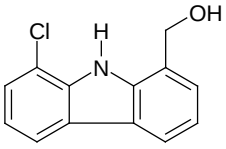
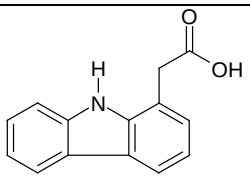
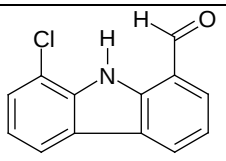
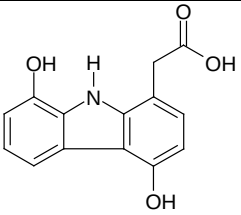
Structure	M.formula and M.weight	Methods of identification	Reference
 8-chloro-9H-carbazole-1-acetic acid	$C_{14}H_{10}ClNO_2$ 259	GC/MS/CI, HPLC/MS, High resolution EI/MS, ^{1}HNMR	Moore et al. (1990)
		GC/MS/EI	Buser et al. (1998b)
		GC/MS/EI, HPLC/MS	Encinas et al. (1998b)
		GC/MS/EI	Poiger et al. (2001)
		LC/TOF/MS	Agueera et al. (2005)
		GC/MS/EI	Scheurell et al. (2009)
 8-hydroxy-9H-carbazole-1-acetic acid	$C_{14}H_{11}NO_3$ 241	GC/MS/CI, HPLC/MS, High resolution EI/MS, ^{1}HNMR	Moore et al. (1990)
		GC/MS/EI, HPLC/MS	Encinas et al. (1998b)
		GC/MS/EI	Poiger et al. (2001)
		LC/TOF/MS	Agueera et al. (2005)
 8-chloro-9H-carbazole-1-methanol	$C_{13}H_{10}ClNO$ 231	LC/MS/MS	Encinas et al. (1998b)
		LC/TOF/MS	Agueera et al. (2005)
	$C_{14}H_{11}NO_2$ 255	GC/MS/CI, HPLC/MS, High resolution EI/MS, ^{1}HNMR	Moore et al. (1990)
		GC/MS/EI	Buser et al. (1998b)
		GC/MS/EI	Vogna et al. (2004)
	$C_{13}H_8ClNO$ 229	LC/MS/MS	Encinas et al. (1998b)
		LC/TOF/MS	Agueera et al. (2005)
		GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
	$C_{14}H_{11}NO_4$ 257	LC/TOF/MS	Agueera et al. (2005)

Table 2: Continued

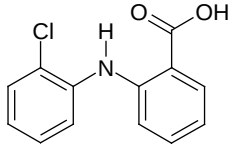
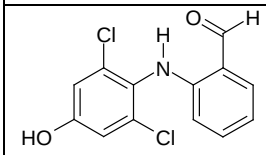
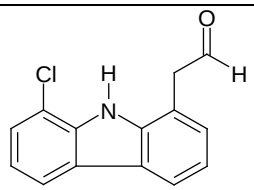
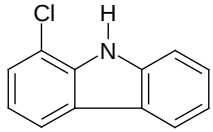
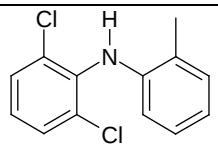
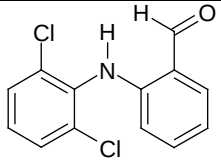
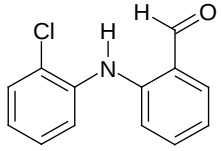
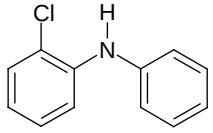
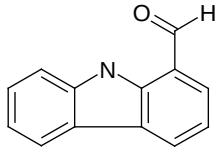
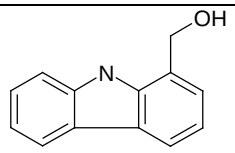
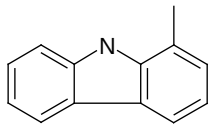
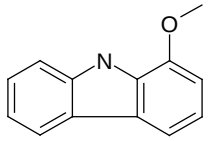
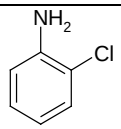
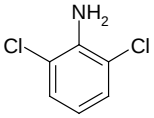
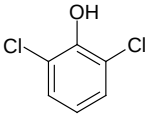
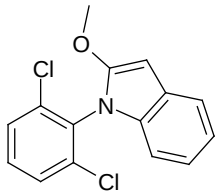
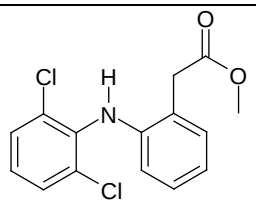
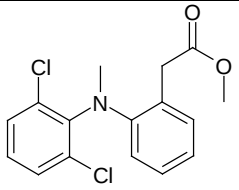
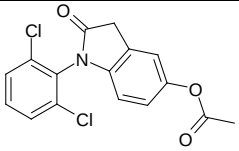
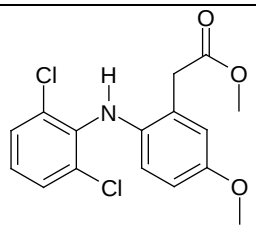
Structure	M.formula and M.weight	Methods of identification	Reference
	$C_{13}H_{10}ClNO_2$ 247	GC/MS/EI	Agueera et al. (2005)
	$C_{13}H_9Cl_2NO_2$ 281	LC/TOF/MS	Agueera et al. (2005)
	$C_{14}H_{10}ClNO$ 243	GC/MS/EI	Agueera et al. (2005)
	$C_{12}H_8ClN$ 201	GC/MS/EI	Agueera et al. (2005)
	$C_{13}H_{11}Cl_2N$ 251	GC/MS/EI	Agueera et al. (2005)
		GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
	$C_{13}H_9Cl_2NO$ 265	GC/MS/EI	Agueera et al. (2005)
		GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
	$C_{13}H_{10}ClNO$ 231	GC/MS/EI	Agueera et al. (2005)
		GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
	$C_{12}H_{10}ClN$ 203	GC/MS/EI	Agueera et al. (2005)
	$C_{13}H_9NO$ 195	GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
		GC/MS/EI, HPLC/MS	Encinas et al. (1998b)
	197 $C_{13}H_{11}NO$	GC/MS/EI, HPLC/MS	Encinas et al. (1998b)

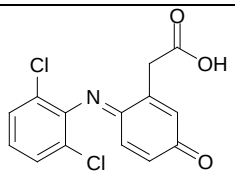
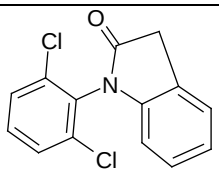
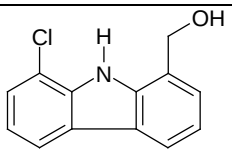
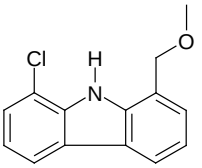
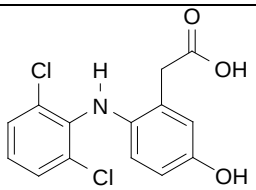
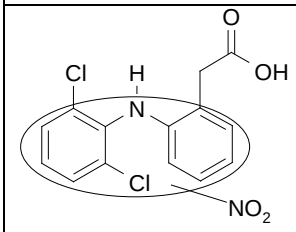
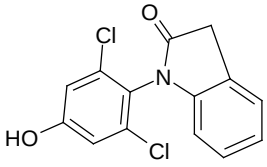
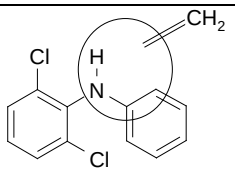
Table 2: Continued

Structure	M.formula and M.weight	Methods of identification	Reference
	181 $C_{13}H_{11}N$	GC/MS/EI, HPLC/MS	Encinas et al. (1998b)
	197 $C_{13}H_{11}NO$	GC/MS/EI, HPLC/MS	Encinas et al. (1998b)
	C_6H_6ClN 127	GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
	$C_6H_5Cl_2N$ 161	GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
		GC/MS/EI	Vogna et al. (2004)
	$C_6H_4Cl_2O$ 162	GC/MS/EI	Bartels and Von Tuempling Jr. (2007)
	$C_{15}H_{11}Cl_2NO$ 292	GC/MS	Pfleger et al., 1992
	$C_{15}H_{13}Cl_2NO_2$ 310	GC/MS	Pfleger et al., 1992
	$C_{16}H_{15}Cl_2NO_2$ 324	GC/MS	Pfleger et al., 1992
	$C_{16}H_{11}Cl_2NO_3$ 336	GC/MS	Pfleger et al., 1992
	$C_{16}H_{15}Cl_2NO_3$ 340	GC/MS	Pfleger et al., 1992

1.1.8 Biotransformation

Biotransformation is a physiological process in which chemical substances are modified or degraded by means of living organisms such as human, bacteria, algae, etc. As organic substances, the biological transformation of pharmaceuticals in the ecosystem, which mainly occurs due to the bacterial action, involves two metabolic pathways: First, metabolism in which bacteria use the compound as a primary carbon source. Second, co-metabolism in which bacteria can partially convert a substance by an enzyme produced during the metabolism of another substance without using it as primary carbon source. In a trace level, substances are most probably metabolized by co-metabolism. The biotransformation products of diclofenac detected in the environment are almost the same products excreted by human and animals, since bacteria and fungus (*Epicoccum nigrum*) were reported to convert diclofenac to its hydroxylated metabolites [Ibrahim et al., 1996; Webster et al., 1998]. The first data dealing with the biotransformation of diclofenac in mammalian excretion were obtained using either diclofenac [Riess et al., 1978] or ^{14}C-diclofenac [Stierlin et al., 1979]. However, there was no indication for the presence of the diclofenac human metabolites in the environment [Buser et al., 1998b, Tixier et al., 2003]. Compared to their laboratory experiments, several authors excluded biotransformation of diclofenac under the environmental conditions not only in surface water [Buser et al., 1998b, Tixier et al., 2003, Kunkel and Radke, 2008] but also in presence of activated sludge [Buser et al., 1998b, Quintana et al., 2005, Joss et al., 2006]. However, in 2007, a major reactive metabolite (p-benzoquinone imine of 5-hydroxydiclofenac) was detected, in a laboratory study, as a result of transformation of diclofenac by the indigenous microflora in river sediments under aerobic conditions [Groning et al., 2007]. They assigned, furthermore, abiotic adsorption to be the further fate for this reactive product. Additionally, in a specially designed small-scale pilot wastewater treatment plant, indolinone, alcoholic, and methoxy derivatives of diclofenac were identified by GC/MS [Kosjek et al., 2007a, b] while hydroxyl, benzoquinone imine derivative and nitro analogue of diclofenac were identified by means of liquid chromatography-quadrupole time of flight-mass spectrometry (LC/QqToF/MS) [Kosjek et al., 2008]. Furthermore, 4'-hydroxy indolinone, 4'-hydroxy- and 5-hydroxy diclofenac were identified in human urine samples [Stuelten et al., 2008a] as well as, for the first time, in WWTP effluents [Stuelten et al., 2008b, Perez and Barcelo, 2008]. Additionally, other seven biotransformation products of diclofenac were detected in activated sludge flow-through bioreactors by Kosjek et al. (2009). Recently, Scheurell et al. (2009) detected the human metabolites 3'-hydroxy-, 4'-hydroxy- and 5-hydroxydiclofenac in surface water. The structure of some biotransformation products of diclofenac are shown in **Table 3** and the others were previously demonstrated in **Figure 5**.

Table 3: Structures of selected biotransformation products of diclofenac

Structure	M.formula and M.weight	Method of identification	Reference
	$C_{14}H_9Cl_2NO_3$ 309	HPLC/DAD, LC/MS/MS/ESI, NMR	Groning et al., 2007
		LC/QqToF/MS/ ESI	Kosjek et al., 2008
	$C_{14}H_9Cl_2NO$ 277	GC/MS	Kosjek et al., 2007a, b
		LC/QqToF/MS/ ESI	Kosjek et al., 2009
	$C_{13}H_{10}ClNO$ 231	GC/MS	Kosjek et al., 2007a, b
	$C_{14}H_{12}ClNO$ 245	GC/MS	Kosjek et al., 2007a, b
	$C_{14}H_{11}Cl_2NO_3$ 311	LC/QqToF/MS/ ESI	Kosjek et al., 2008
	$C_{14}H_{10}Cl_2N_2O_4$ 340	LC/QqToF/MS/ ESI	Kosjek et al., 2008
	$C_{14}H_9Cl_2NO_2$ 293	LC/MS/MS/ ESI	Stuelten et al., 2008b
	$C_{13}H_{10}Cl_2N$ 249	LC/QqToF/MS/ ESI	Kosjek et al., 2009

1.1.9 Risk assessment

Environmental risk assessment (ERA) procedures for pharmaceuticals have been developed for regulatory purposes in Europe using the same principles already applied in the Europian Technical Guidance Documents (EUTGD) of chemicals [Carlsson et al., 2006a, Grung et al., 2008]. Depending on the existing environmental occurrence and toxicity data from the literature, risk quotient of diclofenac could be calculated according to the following equation:

Risk quotient = PEC/PNEC
where:
PEC: Predicted environmental concentration
PNEC: Predicted no-effect concentration

There are mainly two approaches for calculation of worst case PEC of pharmaceuticals in surface water without considering transformation or degradation. The first depends on consumption rates, removal during sewage treatment, human metabolization and/or type of application. In the literature, this approach has been represented in different forms using different equations: equation 1 [EMEA, 2006, Grung et al., 2008, Carlsson et al., 2006a, Fent et al., 2006, Huschek et al., 2004], equation 2 [Sebastine and Wakeman, 2003, Grung et al., 2008, Ashton et al., 2004, EMEA, 2001, Jones et al., 2002, Stuer-Lauridsen et al., 2000] and equation 3 [Tauxe-Wuersch et al., 2005, Johnson et al., 2007].

PEC = (M x **A x 100**)/ (V x D x **DDD x P x 365**) mg/L (equation 1)

The bold digits and letters are called **Fpen** (the percentage of market penetration of pharmaceutical) which is equivalent to the proportion of the population being treated daily with a specific drug (diclofenac) and it is proposed to be 0.01 (or 1 %) [EMEA, 2006]. However, in Norway it is more than 1 % [Grung et al., 2008].

PEC = [A x (1-R/100)]/ (365 x P x V x D) mg/L (equation 2)
PEC = [A x (1-R/100) x (1-E/100)]/ (365 x P x V x D) mg/L (equation 3)
where:
M: Maximum daily dose consumed per inhabitant (mg/inh d); for diclofenac 150 mg/(inh.d)
A: Predicted amount of drug consumed per year in the relevant geographic area (mg/y)
R: Removal rate (in percentage) during sewage treatment; default value = 0
V: Volume of wastewater produced per inhabitant per day; default value = 0.2 L/(inh.d)
DDD: Defined daily dose values [mg/(inh.d)]; obtained from data reported by WHO (100 mg)

P: Number of inhabitants (population) of the geographic area under consideration (inh)
D: Dilution factor; default value = 10
E: Maximal excretion of the conjugated and unchanged drug in percent; default value = 0
365: Number of days/year
100: The conversion factor for percentage.

In case of assess the worst case concentration, drug metabolism in humans and the removal rate during the wastewater treatment processes should be set to 0.
On the other hand, the second approach (equation 4) depends on the specific measured load for each inhabitant [Letzel et al., 2009].

PEC = (SLI x 1000)/ V x D mg/L (equation 4)
where:
SLI: Specific load for each inhabitant (mg/inh d); obtained from real measurements.

It has to be stated that the previously mentioned equations do not result in an acurrate PEC value as they do not consider the transformation of pharmaceuticals in surface water and water/sediment ecosystem, after discharging with the wastewater effluents. However, the first three equations can produce more realistic results in case of limited biotransformable pharmaceuticals. For diclofenac, equation 4 can be considered the most suitable formula giving more environmental relevant results for PEC due to the following reasons:

1. The use of default values in the first three equations does not reflect realistic environmental concentrations in a specific investigated area.
2. The real amount of consumed diclofenac is unknown, since considerable amount of diclofenac is frequently sold over the counter.
3. The amount of diclofenac excreted without metabolization shows high variation (1 to 25 %).
4. The removal rate (0 to100 %) of diclofenac in WWTP shows inconsistent results.
5. Although the dermal application results in a higher discharge of diclofenac (90 %), the total amount of diclofenac applied to the skin cannot be calculated.

Therefore, it could be helpful for the rough calculation to be followed by real value measurements to obtain more accurate estimation.

On the other hand, predicted no-effect concentration of pharmaceuticals can be calculated from the toxicity data provided by specific toxicity tests. Toxicity is often assessed based on EC_{50}, LD_{50} and/or no observed effect concentrations. Predicted no-effect concentration is

obtained by dividing one of these values to an appropriate assessment factor (AF). According to the field of investigation, the AF are calculated and reported in the corresponding TGD. In case of diclofenac, AF estimated at 10 [EMEA, 2006].

Of course, not all pharmaceuticals have to be subjected to the ERA procedure at the same time, but the priority should be determined according to several factors. The physico-chemical properties, PEC, the environmental fate (degradation, bioaccumulation, and sorption) are considered the main factors controlling the selection of pharmaceuticals for ERA [EMEA, 2006, Carlsson et al., 2006a].

For refinement, substances which have log K_{ow} or P_{ow} above 4.5, PEC value >0.01 µg/L in surface water or known to affect aquatic organisms at concentrations even below 0.01 µg/L should be further investigated for environmental fate and toxicity [EMEA, 2006]. Similar strategies exist in the US [Ankley et al., 2007]. For biodegradability, the test should be initially conducted in the WWTP and the substance which is not readily biodegradable (DT_{90} of >3 days) should be investigated in a water/sediment study. Substance that shows significant shift to the sediment (≥ 10 % after or at 14 days), is expected to exhibit potential risk (unless the substance is readily biodegradable). Bioaccumulation can be indicated by K_{ow} > 1000. Sorption in WWTP can potentially be of concern if K_d value > 10000 L/kg, unless the substance is readily biodegradable. Pharmaceuticals with risk quotient > 1 are expected to have an environmental potential risk or to cause environmental adverse effects. Therefore, they should be further evaluated in the aquatic environment in order to apply the necessary risk reduction measures [EMEA, 2006].

For diclofenac, the risk quotient was found to be < 1 [Woldegiorgis et al., 2007, Ashton et al., 2004, Carlsson et al., 2006a, Huschek et al., 2004, Jones et al., 2002] especially when measured environmental concentrations (MECs) were used [Grung et al., 2008]. Therefore, it is not involved within the first ten pharmaceuticals that they could potentially be of concern in the environment. However, using the PECs (0.48-0.75 µg/L) and PNEC (0.1 µg/L) result in a risk quotient > 1 [Grung et al., 2008]. Additionally, based on PECs in wastewater used in the crop irrigation and PNEC of water and rats, the risk quotient of diclofenac was estimated by Munoz et al. (2009) in the range of 6-27 for soil ecosystem and in the range of 1-7 for predator foods. Furthermore, several authors reported that the PECs (0.14-2.21 µg/L) [Huschek and Krengel, 2002, LUA Brandenburg, 2002; Tauxe-Wuersch et al., 2005, Letzel et al., 2009] and the MECs **[Table1]** of diclofenac in surface waters exceeded the action limit 0.01 µg/L by hundred times in some cases. Nevertheless, in addition to the fact that

diclofenac has a log K_{ow} = 4.5, the ecotoxicity data are considered the most important reason to included diclofenac in almost all risk assessment studies of pharmaceuticals.

1.1.10 Ecotoxicity

For evaluation of chronic toxicity, Schwaiger et al. (2004) and Triebskorn et al. (2004) studied the sublethal toxic effects of diclofenac on fish, rainbow trout (*Oncorhynchus mykiss*), exposed to doses ranging from 1 µg/L to 500 µg/L over a period of 28 days. The histopathological examinations of exposed fishes showed adverse effects such as alterations in the liver, kidney and gills at lowest observed effect concentration (LOEC) of 5 µg/L [Schwaiger et al., 2004] and even at 1 µg/L [Triebskorn et al., 2004, 2007] probably due to bioaccumulation. Based on subchronical exposure studies, Hoeger et al. (2005) estimated the no observed effect concentration (NOEC) for brown trout at 0.5 µg/L and the PNEC value at 5 ng/L. Additionally, acute toxicity tests (4 days) were conducted by Hong et al. (2007) for determination of diclofenac toxic effect on male medaka fish (*Oryzias latipes*). At the environmentally relevant concentration of 1 µg/L, they proposed that diclofenac has the potential to cause cellular toxicity, genotoxicity and estrogenic effects to the exposed fishes as revealed from the elevation of three biomarkers cytochrome P450, p53 and vitellogenin in tissues.

The toxicity was not limited to diclofenac only but it extended to its transformation products also which were found to be more toxic than diclofenac itself. It was reported that the phytotoxicity of diclofenac to the unicellular chlorophyte microalgae (*Scenedesmus vacuolatus*) increased sixfold after 53 h of exposure to sunlight [Schmitt-Jansen et al., 2007]. Photosensitization and hence photobiological adverse effects of irradiated diclofenac were demonstrated by Moore et al. (1990) and Quintero and Miranda (2000). Moore et al. (1990) prove the ability of diclofenac to form singlet $^1O_2$ (reactive oxygen species) and free radical species (generated by homolysis of the photolabile carbon-halogen bond) which may react covalently with several biological targets (lipid, protein and/or DNA) compound leading to an exaggerated toxic effect (especially skin cancer). Based on reactive oxygen species and DNA binding assay, Onoue et al. (2009) were able to predict photogenotoxic risk of irradiated diclofenac. 8-chloro-9-H-carbazole acetic acid, the analogue to the phototoxic drug carprofen, considered the main reason of the photosensitivity responses induced by diclofenac through formation of free radicals [Moore et al., 1990, Encinas et al., 1998b]. Lysis of red blood cells (RBCs) occurs only in the presence of 8-chloro-9H-carbazole acetic acid indicating that chlorine substituent plays an important role in the hemolysis process of the red blood cells [Encinas et al., 1998b]. Furthermore, p-benzoquinone imines and the

hydroxylated metabolites mainly 4'- and 5'-hydroxydiclofenac were discussed to be the prime reason for hepatotoxic adverse effects of diclofenac [Bort et al., 1998, Poon et al., 2001].

Additionally, diclofenac does not occur in the environment as a single contaminant, but as complex mixtures with other pharmaceuticals. Therefore, Cleuvers (2003) studied the synergistic toxicity of diclofenac on the aquatic organisms from different taxonomical classes. They revealed that diclofenac in combination with ibuprofen exerted a much stronger toxic effect than could be seen by each compound individually. Furthermore, the most frequently published ecotoxicological problem in different countries is the death of vulture after scavenging diclofenac treated livestock. Rapid decline of vulture population were reported in India, Pakistan, Nepal and recently Egypt [Arun and Azeez, 2004, Oaks et al., 2004, Green et al., 2004, 2006, Cuthbert et al., 2006, Taggart et al., 2007a, b] probably due to different toxicity mechanisms such as increase the production of reactive oxygen species, interference with uric acid transport [Naidoo and Swan, 2009], inhibition of renal prostaglandins [Meteyer et al., 2005], or damage to renal tubular cell [Ng et al., 2006].

Based on the previously mention information, it can be concluded that diclofenac definitely poses a risk on the ecosystems where it is present. Therefore, diclofenac was recently banned on the Indian subcontinent [Naidoo and Swan, 2009] and classified environmentally as dangerous drug in Sweden [RSMPA, 2004, Carlsson et al., 2006a].

2. Research deficiencies and objectives

After reviewing the literature dealing with diclofenac residues in the environment, several important points have to be taken in consideration:

1- Because of the information deficiency, the fate of diclofenac in water/sediment ecosystems is poorly understood and, up to date, no comprehensive study has been conducted to explain this in details.

2- Although many experiments regarding transformation of diclofenac in wastewater treatment plants have been already done, their results can not readily be transferred to the water/sediment compartments.

3- Although sediment compartment (with its higher density of microorganisms than water compartment) is considered the potential major sink of diclofenac, studies concerning the role of sediments in the elimination of diclofenac contaminants from the aquatic compartments are sparse and sometimes incomplete. Additionally, most of the environmental studies concern mainly with analysis of water and they do not pay a great attention to the sediment. Of course water analysis is important, but it describes the contamination levels over a narrow time window. In contrast, sediment analysis provides information over a wider time range of contamination since it is less sensitive to the environmental changes.

4- The investigation of the environmental fate of a substance is a very complex issue especially under field conditions which is more realistic but in the same time relatively expensive, intensive, many data are difficult to interpret and the environmental conditions are difficult to control. In contrast, laboratory tests are considered more helpful for the explanation of the environmental processes (especially when the test substance is used as a radiolabeled material) because the results are usually comparable and reproducible due to the standardized conditions.

5- Although fate studies is a part of a wide effort aimed at environmental risk assessment of pharmaceuticals residues in the environment, the risk assessment using data on transformation of the substances in water/sediment systems is not considered neither in the literature nor in the guidelines probably due to the lack of information. Therefore, making such data available is very helpful for better risk assessment, when it is necessary.

6- Although several studies concerning identification of transformation products of diclofenac are already existed, products arising from the interaction between diclofenac and water/sediment compartments could not be found. The transformation products may have different biological effect compared to the parent compound. Therefore, their identification is essential not only to predict the fate, the

environmental impact on the ecosystem or even to provide a comprehensive risk assessment on drug residues under the environmental conditions, but also for designing improved treatment technologies for elimination of all trace contaminants.

7- Although several identification methods have been already published, the identification of unknowns in complex matrices stills a difficult issue. It requires the application of analytical tools capable of providing the comprehensive information required to final structural elucidation.

8- Using of only one method is not enough for the identification of the transformation products, since formation of large numbers of unknown compounds with different physico-chemical properties may complicate their separation. Therefore, combination of at least two techniques may provide complementary information that enabled the identification of unknowns. Additionally, absence of the transformation products as authentic standards is the most common problem rendering the identification process more complicated with lower degree of confidence than in presence of reference authentic standards.

In this sense, three approaches were determined to be the major aims of the present work.

2.1 Fate study

In this part, laboratory tests on the fate and behavior of ^{14}C-diclofenac in water/sediment systems were conducted according to the OECD guideline 308 [OECD, 2002a] not only to fill the information gap but also to check the applicability of this test to determine the fate and behavior of photosensitive pharmaceutical compounds using a special irradiation apparatus. The fate of ^{14}C-diclofenac was evaluated according to the mineralization rates, the amount of radioactivity remained in the water phase, the extractable residues and the non-extractable residues in respect to the initially applied radioactivity. Especially in the trapping of ^{14}C-carbon dioxide released during the mineralization of ^{14}C-diclofenac, the technical design of the irradiation apparatus (discontinuous gas exchange) and the biometric flask equipped with soda lime trap (continuous gas exchange), recommended by OECD guideline 308 for testing chemicals, are different. Therefore, an additional laboratory batch system that allows for discontinuous gas exchange to maintain aerobic conditions was applied in short- and long-term degradation tests using different types of sediments (sandy and sandy loam), different incubation conditions (dark and light), different incubation intervals (from 0 up to 100 d), different extraction techniques (solid phase extraction, liquid–liquid extraction, and sequential extraction), and different analytical techniques (RTLC and HPLC) were conducted in order to obtain comprehensive results. Additionally, matrix characterization and

sorption/desorption experiments were involved. By means of this complex simulation test, depletion rates in water and sediment, overall disposition of ^{14}C-residues, mass balances, and characterization of biodegradation products are investigated [Ericson, 2007].

2.2 Identification of transformation products

In this part, several analytical techniques were used for investigation of diclofenac and its transformation products in pure water and water/sediment systems under different laboratory conditions. First, HPLC/UVD and HPLC/DAD were used for screening purposes on UV absorbent phototransformation products of irradiated diclofenac using special irradiation apparatus. Second, GC/MS was applied for determination of the exact origin of the diclofenac artifact. Additionally, derivatized and underivatized samples of irradiated diclofenac were examined by GC/MS for identification of polar and non polar phototransformation products, respectively. Third, a new strategy using HPLC combined with the hybrid triple quadrupole-linear ion trap mass spectrometer system (LC/MS/MS-QTRAP) was applied for the first time in the identification the phototransformation products of diclofenac after irradiation experiments or its biotransformation products after dark experiments. Finally, the concentrations of diclofenac were determined for different irradiation experiments.

2.3 Structural confirmation of the main transformation product

In this part, the most abundant phototransformation product of diclofenac should be isolated by means of preparative reversed-phase column chromatography and semi-preparative HPLC to make it available as authentic reference standard material. Afterwards, the structure was confirmed using 2D ^{1}H- and ^{13}C-NMR analysis.

3. Materials and Methods

3.1 Sampling and storage of native water and sediment

Natural water and sediments were taken from headwater stream at Sandbach catchment area near Hordorf and Schandelah, Lower Saxony, Germany. These investigation sites were up-stream located to the next municipal wastewater treatment plant and thus not affected by any effluent contamination. In order to sample mainly sediment under aerobic conditions [OECD, 2002a], the sampling depth was restricted to 5 cm from the upper layer of the sediment. The sediment was then placed in plastic container and covered with native water. Additionally, surface waters were collected from the same sites in separate containers. Water was filtrated, sediment was wet sieved to < 2 mm, homogenized, combined together in ratio of 3:1 and stored at 4 °C in the dark for 28 days at maximum.

3.2 Matrix characterization

Water/sediment samples were characterized at specific time intervals according to the OECD guideline 308 **[Table 4]**. Several parameter were determined in water and sediment phases such as pH, redox potential (E_h), oxygen content (O_2), water holding capacity (WHC), and substrate induced respiration (SIR). Additionally, the grain size analysis was performed by the Institute of Geoecology, TU Braunschweig.

Table 4: Characterization of water/sediment samples [OECD, 2002a]

Parameter	Stage of test procedure				
	After sampling	Before equilibration	At the start the of test	During test	At the end of the test
Water					
Source	X	---	---	---	---
pH	X	X	X	X	X
Eh	X	X	X	X	X
O_2	X	X	X	X	X
TOC	---	X	X	---	X
Sediment					
Source	X	---	---	---	---
Depth	X	---	---	---	---
pH	X	X	X	X	X
Eh	X	X	X	X	X
TOC	---	X	X	---	X
Microbial activity	---	X	X	---	X

X = performed, --- = not determined, Eh = redox potential, O_2 = oxygen content, TOC = total organic carbon

3.2.1 Determination of water holding capacity

Each sediment type (3 g) was transferred to an aluminum dish, homogenized and equally distributed by a spatula or spoon. The dish was then positioned into an infrared heater with analytical balance (Ultra-X 210, Gronert, Germany). Afterwards, the water was completely evaporated using the infrared heater to constant dry mass. The difference between the weight of the sediment before and after drying was listed. The percentage of water holding capacity (WHC) and the dry substance (ds) were calculated according to the following formulas:

$WHC\ (\%) = [(W_i - W_f)/W_i]x\ 100$

$ds\ (\%) = 100 - WHC\ (\%)$

where:

W_i: initial weight of the sediment before drying

W_f: final weight of the sediment after drying

The test was performed directly after sampling to avoid water losses and repeated four times for each sediment type.

3.2.2 Determination of pH and redox potential

The pH and redox potential values were measured in the water phase by means of pH and redox meter pH Multical 535 GLP equipped with pH-glass electrode and redox-electrode (WTW, Weilheim, Germany). Afterwards, they were measured directly in the wet sediments after decantation of the water phases. The pH meter was calibrated before each measurement using three different buffer solutions (pH 4, 7, 9.2). These solutions were freshly prepared every month. The reading was recorded after getting stable for 5 min. In case of the Eh, the voltage was measured by silver/silver chloride electrode so that the obtained value was added to + 211 mV to be related to the voltage of standard hydrogen electrode and the results were expressed as redox potential Eh. By the same way as the pH, the redox electrode was checked prior each series of measurements by determining their response in the redox buffer solution. The reading was recorded after 30 min from the beginning of measurement.

3.2.3 Determination of the dissolved oxygen content

Oxygen content was measured only in water phase by oxygen-meter Oxi 340i with OxiCell 325 oxygen-electrode (WTW, Weilheim, Germany). The electrode of the instrument was immersed directly in the samples before filtration. After reaching a stable value, the reading was recorded and the oxygen content was expressed in mg/L.

3.2.4 Determination of the total organic carbon

Total organic carbon (TOC) was determined by means of the organic carbon analyser C-Analyzer Dohrmann DC-90 (Dohrmann, Santa Clara, CA, USA). For this purpose, known weight sediment samples were combusted in an oxygen stream at 900 ºC and the released carbon dioxide was subsequently detected by a non-dispersive infrared detector. Preliminary, the sediment samples were treated with an excess of hydrochloric acid (4 M) to remove carbonates and homogenized by gentle shaking. Afterwards, samples were heated on a hot

plate at 100 ºC to remove the excess HCl. Samples were then dried in drying oven at 105 º C overnight. After cooling, samples were ground in a mortar by a pestle for TOC analysis. The TOC amount was calculated on the basis of an external standardization. The standard curves (20 to 150 µg carbon range) were constructed using mixture of oxalic acid dehydrate and aluminum oxide in a ratio (1:9). Finally, the results were expressed in % of dry substance according to the following formula:

$TOC\ (\%) = (W_b/W_a) \times 100$

where:

W_a: initial weight (µg)

W_b: amount of carbon (µg)

In each water sample, on the other hand, TOC was measured after decantation. Prior to the measurements few drops of concentrated HCl were added to each sample to remove carbonate bound carbon. The obtained results were expressed in mg/L.

3.2.5 Determination of the microbial activity

Using OxiTop® OC 110 control system with OxiTop®-C measuring head (WTW, Weilheim, Germany), the microbial activity of the sediments was determined as a function of pressure decrease due to the microbial respiration process initiated by a certain substance. This process is known as substrate induced respiration (SIR). For this purpose, 0.4 g of glucose was added to 50 g (ds) of sediment into each 1 L glass vessel. Afterwards, KOH pellets were put using pincers into rubber quivers and hanged in the vessels in order to adsorb the released carbon dioxide due to the respiration process. Finally, the measuring heads were screwed tightly and the vessels were incubated in the dark at 20 ± 1 ºC for 5 days. The negative pressure developed due to the consumption of the oxygen was recorded and the microbial activity was expressed in mg O_2/(100 g h) [ISO, 1997]. Data regarding characterization of water and sediments directly after sampling are given in **Table 5**.

Table 5: Characteristics of water and sediments directly after sampling

	Water	Sediment		
		S1	S2	S3
Sediment type	----	sand	sandy loam	sandy loam
Sand [%]	----	90	53	45
Silt [%]	----	1.5	20	31
Clay [%]	----	8.5	30	27
TOC	49.3-67.4 mg/L	0.2 % ds	0.4 % ds	0.5 % ds
WHC [%]	---	20	30	40
pH	8.3	7.4	7.3	7.2
Eh [mV]	430	210	220	240
O_2 [mg/L]	7.8	---	---	---
SIR [mg O_2/100 g h]	----	1.85	1.90	---

--- = not determined, TOC = total organic carbon, WHC = water holdig capacity, Eh = redox potential, SIR = substrate induced respiration, ds= dry substance

3.3 Reference standards

3.3.1 ^{14}C-diclofenac standards

The stock standard solution (6037 Bq/µL) and the working standard solutions (1483 Bq/µL and 925 Bq/µL) of phenylacetic acid ring-U-^{14}C-diclofenac, specific radioactivity 2260 MBq/ mmol (Amersham Pharmaceutical Biosciences, Little Chalfont, and Buckinghamshire, UK) were prepared in methanol. The structure and labelling position of ^{14}C-diclofenac are shown in **Figure 8**.

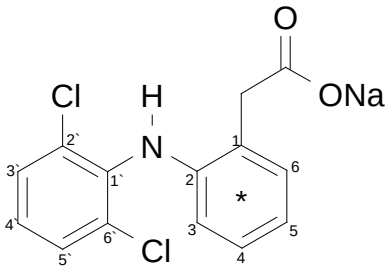


Figure 8: Chemical structure of ^{14}C-diclofenac sodium showing the labeling position

The radiochemical purity (85 %) was checked by means of radio-thin layer chromatography (RTLC) as described for the screening of transformation products (cf. chapter 2.7.8). Only limited amount of ^{14}C-diclofenac standard solution was available for the fate monitoring study. Therefore, an additional clean up procedure could not be conducted.

3.3.2 Diclofenac standards

In 10 mL volumetric flasks, diclofenac (Sigma-Aldrich, Deisenhofen, Germany) stock standard solutions at concentration of 1 µg/µL were prepared in methanol, acetone, and pure water and at concentration of 0.1 µg/µL in ethyl acetate (after ultrasonication for 15 min) while working standard solutions were prepared at concentration of 15 ng/µL in acetone, and 10 ng/µL in methanol and pure water. All prepared standard solutions were stored at – 20 °C except that were prepared in pure water (stored at 4 °C).

3.4 Determination of sorption, desorption and isotherms

By means of sorption or distribution coefficients (K_d), the affinity of ^{14}C-diclofenac to the water or the sediment phase can be asseseed. The K_d was determined according to the OECD guideline 106 [OECD, 2002b]. For this purpose, sediment S3 was air dried and then sieved to particle sizes <2 mm. A 0.01 M $CaCl_2$ aqueous solution was used as a solvent phase. Afterwards, the sediment (20 g) and the $CaCl_2$ solution (60 mL) were mixed, in a PTFE capped centrifugation tubes, in the ratio of 1:3. This ratio was preliminary selected to simulate the same ratio used during the fate study. The mixtures were then equilibrated under constant agitation on a horizontal shaker at 230 rpm for 24 h. Afterwards, different volumes (concentrations) of working standard solution of ^{14}C-diclofenac in methanol (1828 Bq/µL) were spiked directly into the aqueous phase (the volumes of methanol were less than 0.1 % (v/v) of total solution volume). The number of replicates for each concentration was four. After shaking the mixtures for 24 h, the aqueous phases were separated by centrifugation (Centrifuge; Megafuge 1.0, Heraeus GMbH, Germany) at 3000 rpm for 5 min

to remove particles with a diameter greater than 0.2 µm from aqueous phase. The amounts of ^{14}C-diclofenac sorbed onto the sediment were calculated as the difference between the amount ^{14}C-diclofenac initially applied in the solution and the amount remained at the end of the agitation period (indirect method). Preliminary, centrifugation tubes containing only $CaCl_2$ solution spiked with ^{14}C-diclofenac showed no considerable losses during the experiments. For comparison purpose, surface water was used as a solvent phase instead of $CaCl_2$ to determine the K_d value at single concentration level. A blank for each concentration was made as specified by OECD guideline 106 [OECD, 2002b].

Finally, the K_d was determined according to equation number 1. It describes the ratio between the mass concentration of the ^{14}C-diclofenac in the sediment phase and the mass concentration of the ^{14}C-diclofenac in the aqueous solution, under the test conditions, when sorption equilibrium is reached.

$$K_d = C_s/C_w = (m_s \times v_o)/(m_w \times m_{sed}) \quad (1)$$

where:

C_s: content of the ^{14}C-diclofenac adsorbed on the sediment at sorption equilibrium (µg/kg).

C_w: mass concentration of ^{14}C-diclofenac in the aqueous phase at sorption equilibrium (µg/L).

m_s: mass of the ^{14}C-diclofenac adsorbed on the sediment at sorption equilibrium (µg).

m_w: mass of the ^{14}C-diclofenac in the solution at sorption equilibrium (µg).

m_{sed}: quantity of the sediment phase, expressed in dry mass of sediment (kg).

v_o: initial volume of the aqueous phase in contact with the sediment (L).

Furthermore, the influence of concentration on the sorption process was tested by the determination of Freundlich and Langmuir sorption isotherms. Six different concentrations of ^{14}C-diclofenac ranging from 0.06 to 0.6 Bq/L were applied. The sorption data were found to be fitted to the Freundlich equation which relates the amount of the ^{14}C-diclofenac sorbed onto the sediment to the concentration of the ^{14}C-diclofenac in solution at equilibrium (equation 2). This equation was used in its logarithmic or linear form (equation 3).

$$C_s = K_F \times C_w^{1/n} \quad (2)$$

where:

C_s: content of ^{14}C-diclofenac sorbed on the sediment at sorption equilibrium (µg/kg).

C_w: mass concentration of ^{14}C-diclofenac in the aqueous phase at sorption equilibrium (µg/L).

K_F: Freundlich sorption coefficient ($\mu g^{1-1/n}\ L^{1/n}/kg$).

1/n: Freundlich exponent, Freundlich sorption capacity parameter, Freundlich affinity constant or Freundlich regression constant.

Logarithmic form: $\log C_s = \log K_F + 1/n \log C_w$ (3)

Freundlich sorption coefficient K_F ($\mu g^{1-1/n}$ $L^{1/n}$/kg) is the amount of ^{14}C-diclofenac (µg) adsorbed per one kg sediment after the equilibrium had been established. It can be calculated from the intercept value of the Freundlich isotherm. The Freundlich exponent (Freundlich sorption capacity parameter) 1/n is unitless [Scheytt et al., 2005a], sometimes also n is used [Ramil et al., 2010, Barron et al., 2009, Chefetz et al., 2008]. It is equal the slop of the Freundlich isotherm. 1/n describes the affinity of ^{14}C-diclofenac to the sediment (the higher 1/n the higher affinity to the sediment).

Since desorption process also plays an important role in the behavior of a chemical in the environment, desorption experiments were preformed by replacing the $CaCl_2$ solutions with fresh solutions. The new mixtures were agitated for 24 h. Afterwards, the amount of ^{14}C-diclofenac desorbed from the sediment was determined in the aqueous phase. Desorption was calculated as the percentage of the amount of ^{14}C-diclofenac which is desorbed related to the amount of diclofenac previously adsorbed. The purpose of this experiment is to investigate whether diclofenac is reversibly or irreversibly adsorbed on sediment. Desorption is considered reversible when the amount desorbed is more than 75 % of the amount adsorbed [OECD, 2002b]. The desorption coefficient (K_{des}) was determined according to equation number 4. It describes the ratio between the amount of ^{14}C-diclofenac remained in the sediment phase and the amount of ^{14}C-diclofenac desorbed in the aqueous solution, when desorption equilibrium is reached.

$K_{des} = [(m_s - m_w) \times v_o] / m_w \times m_{sed}$ (4)

where:

m_s: mass of the ^{14}C-diclofenac adsorbed on the sediment at sorption equilibrium (µg)

m_w: mass of the ^{14}C-diclofenac desorbed from the sediment at desorption equilibrium (µg)

m_{sed}: quantity of the sediment phase, expressed in dry mass of sediment during the desorption experiment (kg)

v_o: total volume of the aqueous phase in contact with the sediment during the desorption experiment (L).

Furthermore, Freundlich desorption isotherm ($K_{F\text{-}des}$) was calculated according to equation number (5). The Freundlich desorption isotherms equation relates the amount of ^{14}C-diclofenac remaining adsorbed on the sediment to the amount of ^{14}C-diclofenac in solution. Desorption data were found to be fitted to the logarithmic form of the Freundlich desorption equation (equation 6).

$$C_s = K_{F\text{-}des} \times C_w^{1/n} \qquad (5)$$

C_s: amount of ^{14}C-diclofenac remaining sorbed on the sediment at the desorption equilibrium (µg/kg);

C_w: amount of diclofenac in the aqueous phase at the desorption equilibrium (µg/L)

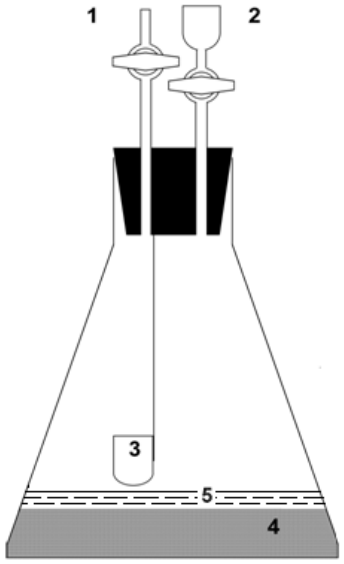
$$\text{Logarithmic form: } \log C_s = \log K_{F\text{-}des} + 1/n \log C_w \qquad (6)$$

3.5 Water/sediment tests in the dark

For the biotransformation tests of ^{14}C-diclofenac, 300-mL Erlenmeyer flasks filled with 50 g (ds) sediments (S1 or S2) and 150 mL surface waters were prepared. The water sediment systems were then equilibrated, without stoppers, at 20 ± 2 ºC for 7 days to reach reasonable stability of the system. After the equilibration period, aliquots (50 µL each) of ^{14}C-diclofenac were spiked directly into the water phase of the test systems using water-miscible organic solvent, e.g., methanol. The concentration of the organic solvent in the overlying water did not exceed 0.5 %. The spiking level of the ^{14}C-diclofenac was 0.07 MBq giving a concentration of 200 µg/kg sediment or 70 µg/L water. The adjustment of the applied amount of the radiotracers focused on the diclofenac relative environmental concentration as well as on the analytical feasibility mainly defined by the specific radioactivity. After fortification, the flasks were then gently shaken (manually) disturbing the sediment as little as possible. Flasks regarding each type of the sediment were then divided into two main groups in order to differentiate between mineralization (MIN), ^{14}C-diclofenac residues in the aqueous phase (AP), in the extractable fraction (ER) and non-extractable fraction (NER) using two different biodegradation systems.

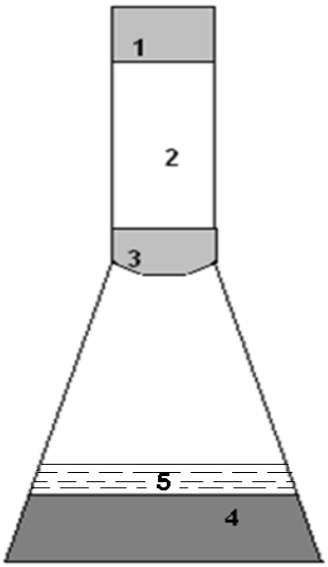
First, the laboratory-batch system **[Figure 9]** in which the flasks were equipped with an inlet and an outlet valve allowing for discontinuous gas exchange [Kreuzig et al., 2003] was applied. Additionally, it is equipped with an internal trap filled with 8 mL 0.1 M potassium hydroxide solution to absorb ^{14}C-carbon dioxide, potentially released by mineralization.

Second, the biometric flask system **[Figure 10]** was applied. It was equipped with a tube filled with 20 g granulated soda lime (Merck, Darmstadt, Germany). The tube was coupled with the flask to absorb ^{14}C-carbon dioxide that might be released by mineralization. This system allows for continuous gas exchange. Therefore, aerobic condition was maintained all the time.



1: inlet valve, 2: outlet valve with activated charcoal filter, 3: internal ^{14}C-carbon dioxide absorption trap (8 mL 0.1 M potassium hydroxide solution), 4,5: water/sediment sample

Figure 9: Laboratory-batch system (discontinuous gas exchange system) for biotransformation tests of ^{14}C-diclofenac in water/sediment systems



1, 3: glass wool, 2: soda lime, 4,5: water/sediment sample

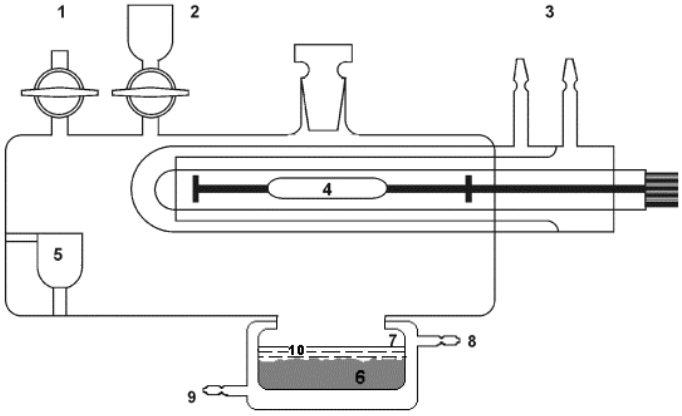
Figure 10: Biometric flask system equipped with soda lime trap (continuous gas exchange) for aerobic biotransformation tests of ^{14}C-diclofenac in water/sediment systems

Afterwards, the test systems of each sediment type were incubated in the dark at 20 ± 2 ºC for different intervals (0, 1, 3, 7, 14, 28, 56, 100 d) and (3, 56, 100 d) in case of both laboratory test systems, respectively. Additionally, in the batch systems, the potassium hydroxide trap and the air above the water phase were exchanged every 4 days to improve the trapping capacity of the alkaline solution and to save aerobic conditions.

For matrix characterization during the test period, water/sediment samples were prepared as mentioned before. These samples were fortified with non-labeled diclofenac (200 µg/kg sediment or 70 µg/L water). To achieve comparability, the samples were incubated parallel to the ^{14}C-diclofenac tests. Both laboratory test systems were equipped with the corresponding ^{14}C-carbon dioxide traps, although they would not allow for determination of mineralization. Additionally, blank water/sediment samples before equilibration, after equilibration, and at the termination of the experiment were conducted as control samples. In order to test the effect of methanol (in which ^{14}C-diclofenac was dissolved) on the microbial activity of the sediments, moreover, water/sediment samples (solvent control) were spiked with 50 µL, each, of pure methanol were prepared as mentioned before. Approximately, 120 water/sediment tests were performed during this investigation.

3.6 Irradiation experiments

To examine the photoinduced effect on diclofenac in water/sediment system, a special irradiation apparatus **[Figure 11]** was used [Kreuzig, 1998, Höllrigl-Rosta et al., 1999, Kreuzig et al., 2003, Kreuzig and Höltge, 2005]. It consisted of a sample container provided by a cooling system and a glass tube with inlet and outlet valves, containing the mercury medium-pressure lamp (Schott, Mainz, Germany). The lampe is surrounded by Pyrex® cooling jacket defining the cut off at λ = 290 nm. The power supply is connected to a timer clock to get the required irradiation periods.



1, 2: inlet-/ outlet valve with activated charcoal filter 3: cooling device, 4: mercury medium pressure lamp with Pyrex® cooling jacket (cut off: λ = 290 nm), 5: ^{14}C-carbon dioxide absorption trap, 6: sediment 7: exchangeable sample container, 8,9: water cooling device, 10: water

Figure 11: Irradiation apparatus for testing on photoinduced transformation

3.6.1 Water/sediment tests

For each sediment type, the water/sediment samples were prepared and equilibrated as mentioned under the biotransformation test (cf. chapter 3.5). In contrast to the biotransformation test, the content of the flask was carefully transferred after the equilibration time into the sample container of the irradiation apparatus and left for a certain period to obtain clear water phase as much as possible. Then, 50 µL ^{14}C-diclofenac were spiked directly into the water phase of the test system. An internal potassium hydroxide trap was used to absorb ^{14}C-carbon dioxide. After fixing the sample container tightly into the irradiation apparatus, the incubation was started at 20 ± 2 °C under 10 h-light/14 h-dark for the 3-day incubation period.

Furthermore, in order to differentiate between biotic and abiotic transformation processes, additional experiments using pure water/quartz sand instead of real water/sediment samples were performed [Kunkel and Radke, 2008]. The quartz sand was preliminary tested for microbial activity by means of SIR method (cf. chapter 3.2.5) within 3 days incubation period. The results showed no sign of any respiration process indicating that the samples were sterile enough. Other methods for sterilization such as autoclavation, addition of sodium azide, mercuric chloride or formalin were excluded because they may lead to structural alteration and may influence the sorption properties of the natural sediment [OECD 2002a, Lotrario et al., 1995, Ramil et al., 2010].

3.6.2 Water tests

In order to estimate the role of the sediment in the removal of the transformation products of diclofenac, irradiation tests were additionally conducted using native water and demineralized water separately without sediment. For this purpose, 150-mL water samples were fortified with 50 µL ^{14}C-diclofenac each and incubated under the same irradiation conditions (cf. chapter 3.6.1). The obtained results were then compared with the irradiation experiments performed in water/sediment system.

For quality assurance, all water/sediment samples prepared during the whole experiments were conducted in duplicate. Additionally, the radioactivities were measured in the extracts applying different volumes at different extraction steps. Finally, the mass balances were conducted allowing for a comparison of the quantity of radioactivity present in the each compartment against the quantity initially applied into the test system. The results were accepted when the recovery rates were in the range of 90 % to 110 % and the standard deviations did not exceed 10 %. In all presented figures, the error bars show the mean standard deviation between the two tests, their lower and their upper ends refer directly to the individual measurement values.

3.7 Radiotracer analysis

At the termination of each incubation interval, the materials (potassium hydroxide solution or the soda lime granules) for ^{14}C-carbon dioxide trapping were removed. Then, the water/sediment systems were separated for analytical purposes. If necessary, sediment and water samples were stored at -20 or 4 °C, respectively, until analysis.

3.7.1 Determination of mineralization rates

Du to the mineralization processes, the released ^{14}C-carbon dioxide can be measured in different ways according to the type of ^{14}C-carbon dioxide trap. In the laboratory-batch system, the alkaline absorption solution was removed by means of a syringe with a long needle and transferred into a vial containing 10 mL Quicksafe A (Zinsser, Frankfurt, Germany). In the biometric flask system, the ^{14}C-carbon dioxide sorbed to the soda lime had to be transferred quantitatively into the liquid phase for measurement. For this reason, the soda lime granules were mixed with 60 mL of hydrochloric acid and stirred until complete dissolution. Nitrogen was introduced gently through the soda lime/hydrochloric acid mixture. The ^{14}C-carbon dioxide in the resulting gas flow was sequentially trapped in 3 successive vials containing 15 mL of Oxysolve-C400 each (Zinsser, Frankfurt, Germany). In both cases, the vials were then analyzed by the liquid scintillation counter (LSC; Wallac 1409, Turku,

Finland) for 30 min using background correction. Later on, the mineralization percentage was calculated according to the following equation:

MIN = A1 x 100/A2

where:

A1: Radioactivity in the analyzed absorption solution (Bq).

A2: Initially applied radioactivity (Bq).

100: Conversion factor to percent.

Principles of liquid scintillation counting

The liquid scintillation counter is a very precise, simple, and fast instrument for measuring radioactivity in liquid samples. It is mainly composed of a dark chamber with 2 photomultiplier tubes and a pulse counter. Hence, this instrument is designed to measure light energy in the dark. Therefore, the radioactive emissions should be converted to photons of light in order to be detected by the photomultiplier tube. The radioactive emissions include alpha particles (α), beta particles (β), and gamma rays (γ). Radiocarbon atoms of the ^{14}C-labeled substances are characterized by neutron rich nucleoli that decay with a half-life of 5730 years. During the decaying processes, neutrons are converted to a protons resulting in emission of β particles and neutrinos. The latter are almost impossible to detect by LSC as they do not interact significantly with matter. The process of beta decay of ^{14}C-substances can be demonstrated by the following reaction:

$$^{14}_{6}C \xrightarrow{\text{decay}} {}^{14}_{7}N + e^{-} (\beta \text{ particles}) + \text{neutrino}$$

In comparison with gamma rays, beta particles have low penetration power and can not be directly detected by the instrument. For this purposes, the radioactive samples is placed in a vial containing a relatively dense scintillation cocktail either for aqueous solvents (Quicksafe A) or for organic solvents (Quicksafe N). Both cocktails consisted of solvent and fluor (scintillator compound that fluoresces when it is bombarded with β particles). The role of solvent is to efficiently absorb the bulk of the radiation energy produced by beta particles before all of its kinetic energy is dissipated by some other mechanisms (heat or ionization). Because of their high electron density, aromatic compounds (benzene derivatives) such as toluene and xylene and the less toxic phenyl xylylethane were found to be the best solvents for LSC. Up on absorption of beta energy, the solvent molecules are converted to the exited state. However, these compounds have little tendency to emit light and hence could not be detected. In order to return to the ground state, alternatively, the exited solvent molecules

transfer the captured energy to other solvent molecules which are the fluors. The later are broadly divided into primary, e.g. 2,5-diphenyloxazole, and secondary, e.g. p-bis-(o-methylstyryl) benzene, scintillators. The role of the primary scintillator is to receive the energy of the solvent molecules and to emit it in the form of light flashes at a wavelength that could be detected by the photomultiplier tubes. However, the early photomultiplier tubes were found to be not sensitive in the region of fluorescence emission of the primary scintillators as most of them were found to emit light below 408 nm. Therefore, the secondary scintillators or the wavelength shifters were introduced to capture the flashes of the excited primary scintillators and emit it at longer wavelength region that more suitable to the photomultiplier tubes. The latter convert the flashes of light into countable electrical pulses and record them as counts per minute (cpm). Since the scintillation counter is not 100 % efficient, the cpm should be converted to disintegrations per minute (dpm) in order to get the number of decay which actually occurred. The dpm is then converted to the systematic international unit of radiation, becquerel (Bq), which corresponds to 60 dpm.

On the other hand, the energy emitted by beta particles or photons during the energy transfer process is not only absorbed by the scintillation cocktail but also by sample components leading to losses of energy. This process is known as quenching which can easily decrease the fluorescence intensity and hence reduce the pulses below the detection limit of the counter, thus reducing the overall counting efficiency. Three types of quenching are mainly known, physical, chemical and colour. For quench correction, the external artificial gamma emission source Europium-152 (^{152}Eu), radioactivity of 0.74 MBq, was applied next to the sample vial and after the initial counting, the sample is counted again. Gamma rays result in emission of Compton electrons which show the same quench effects as the sample spectrum. For determination of the extent of quenching, therefore, the measured energy spectrum of the Compton electrons is compared with their theoretical energy spectrum.

3.7.2 Determination of ^{14}C-diclofenac residues in water phases

At the termination of each incubation interval, the water/sediment systems were separated by decantation and filtration, with minimal disturbance to the sediment as it was described by Ericson (2007), Löffler et al. (2005), OECD (2002a). By means of graduated measuring cylinder, the total water volume was then determined. Afterwards, 3 different aliquots (50, 100 and 500 µL) were mixed individually with Quicksafe A (10 mL in a vial) and the radioactivities were measured by LSC for 30 min using background correction.

It was the objective not to disturb the water/sediment interface assumed to be the most important layer for transformation process [Craven et al., 1986]. Therefore, any rinsing of the sediment samples was avoided durig the test period considering that the concentration of ^{14}C-diclofenac in the pore water is the same as in the water phase. Therefore, the radioactivity amount of the pore waters was added to the extractable fraction (cf. 3.7.4).

However, additional experiments were performed to estimate the radioactivity amount that could be retained in the pore waters. For this purpose, laboratory-bach water/sediment systems were prepared as mentioned before (cf. 3.5) and incubated in the dark for 7 days. After separation of water/sediment system as mentioned here, some of the sediment samples were rinsed with 3 x 50 mL fresh water to exchange the pore water. Afterwards, the radioactivity in the total volume of the pooled water was measured. On the other hand, the other sediment samples were extracted directly with ethyl acetate (cf. 3.7.4). Finally, the results of both extraction methods were compared for determination of the effect of pore water. The radioactivity % in the aqueous phase (AP) was calculated according to the following equation:

AP = A1 x V2 x 100/A2 x V1
where:
A1: Radioactivity in the analyzed aliquot (Bq).
A2: Initially applied radioactivity (Bq).
V1: Volume of the analyzed aliquot (mL).
V2: Total volume of the measured water phase (mL).
100: Conversion factor to percent.

3.7.3 Determination of DT_{50} and DT_{90} in water phases

The dissipation time DT_{50} and DT_{90} are the times at which the initial concentration of ^{14}C-diclofenac is reduced from the water phase to 50 and 90 %, respectively. Based on the residual radiotracer amounts in the water phase, the dissipation times DT_{50} and DT_{90} were calculated using the ModelMaker (2007) statistical software [Moenickes, 2010]. The regression curves were calculated from first order kinetics depending on best fit of at least 5 points. DT_{50} and DT_{90} can be calculated also using the elimination rate constant (k_{elim}) according to the following equations:

$DT_{50} = \ln 2/k_{elim}$ (1)

$DT_{90} = \ln 10/k_{elim}$ (2)

3.7.4 Determination of ^{14}C-diclofenac residues in extractable fractions

After separation of the water/sediment systems, the sediments were extracted individually with 150 mL of ethyl acetate using a horizontal shaker at 200 rpm overnight for determination of the extractable residues (ER). The suspensions were then decanted through folded filters and the sediment was rinsed three times with 50 mL of ethyl acetate. The total volume of the pooled ethyl acetate was measured and the radioactivities in 2 (50 and 100 µL) aliquots were determined, after mixing with Quicksafe N (10 mL), by LSC for 15 min using background correction. Afterwards, the ethyl acetate extracts were evaporated by means of rotary evaporator to 5 mL. The radioactivity in 10 µL of the concentrated extracts was then determined. Afterwards, the extracts were stored in brown glass vials at 4 ºC until screening analysis.

The radioactivity % in the ER was calculated according to the following equation:

ER = A1 x V2 x 100/A2 x V1

where:

A1: Radioactivity in the analyzed aliquot (Bq).

A2: Initially applied radioactivity (Bq).

V1: Volume of the analyzed aliquot (mL).

V2: Total volume of the organic solvent (mL).

100: Conversion factor to percent.

For quality assurance, the extraction efficiency of ethyl acetate against acetone, acetonitrile and methanol was preliminary tested. For this reason, in 300-mL Erlenmeyer flasks, 50 g (ds) wet sediment (S3) was applied and spiked with ^{14}C-diclofenac at approximately 1.7 MBq/kg each. Afterwards, sediments were extracted individually with the corresponding solvent as mentioned before for ethyl acetate. Furthermore, 50 g sediment samples (dry substance content) were spiked with aqueous diclofenac standard at 1 or 2 mg/kg. The diclofenac was then extracted by means of ethyl acetate as mentioned above and analyzed using HPLC/UVD. The recoveries of diclofenac were found to be 98 ± 5 %.

3.7.5 Determination of ^{14}C-diclofenac residues in non-extractable fractions

For the determination of non-extractable residues (NER), the extracted sediments were transferred onto evaporating dishs and left for drying. The sediments were then

homogenized with mortar and pestle. To minimize the variability, 4 aliquots (150-200 mg sediment) of each sediment sample were mixed with 10 mg cellulose (Merck, Darmstadt, Germany) in the sample boats and burned for 4 min in the oxygen stream (350-380 mL/min) at 900 ºC of the biological oxidizer (OX-500, Harvey Instruments Hillsdale, NJ, USA). The resulting ^{14}C-carbon dioxide was trapped in vials containing Oxysolve-C400 (15 mL). The radioactivity was then determined by LSC for 10 min without background correction. Later on, the sediments were stored in brown bottles at - 20 ºC.

To control the performance of the instrument, recovery rates of the sample oxidizer were determined before starting, during and at the end of the measurements. For this reason, 3 blank sediment samples were prepared as mentioned above and spiked with a defined amount of the reference standard ^{14}C-LU-111995 ((+)-exo-{2-[6-(4-fluorophenyl)-3-aza-bicyclo[3.2.0] heptane-3-yl]ethyl}-1,3H-chinazoline-2,4-doin-fumerate; Knoll, Ludwigshafen, Germany) and combusted directly after the preheating time. After burning of each spiked sample, blank sediment was burned in order to avoid memory effects. Furthermore, after each set of sample replicates one blank, one standard and another blank were combusted. Additionally, a ^{14}C-standard paper with radioactivity 5000 dpm was burned at the end.
The radioactivity % in the NER was calculated according to the following equation:

NER = A1 x M2 x 100/A2 x M1
where:
A1: Radioactivity in the analyzed aliquot (Bq).
A2: Initially applied radioactivity (Bq).
M1: Weight of the analyzed aliquot (g).
M2: Total weight of the dried sediment sample (g).
100: Conversion factor to percent.

3.7.6 Chemical characterization of non-extractable residues

At the termination of both dark and irradiation water/sediment experiments, the matrix affinity of non-extractable residues to the sediment was tested sequentially by second and third extraction steps. The first step was performed according to Heise et al. (2006). For this purpose, 150 mL methanol and 600 µL of hydrochloric acid were added to 20 g of dried, ethyl acetate extracted, sediments in 300-mL Erlenmeyer flasks. The flasks were then shaken on a horizontal shaker at 220 rpm overnight. The extracts obtained after filtration were rotary evaporated to 5 mL. Aliquots were taking before and after evaporation, mixed with Quicksafe N (10 mL) each, and subsequently scintillation counted. After drying, the radioactivities in the

methanol/hydrochloric acid extracted sediments were analyzed for the non-extractable residue as mentioned under 3.7.4. As a third step of the sequential extraction procedure, a silylation technique, for derivatization of humic substances using silylation reagents at room temperature, was applied [Haider et al., 1992, Klaus et al., 1998 Oesterreich et al., 1999]. For this purpose, 15 g of the dried, methanol/hydrochloric acid extracted, sediments were filled into centrifuge tubes and treated with 40 mL N-dimethylformamide and 5 mL chlorotrimethylsilane. After shaking overnight at 220 rpm, the suspensions were centrifuged (Megafuge 1.0, Heraeus GMbH, Hanau, Germany) at 3000 rpm for 5 min and the organic supernatants were then filtered, the moist sediment retreated with 10 mL N-dimethylformamide, centrifuged for 5 min at 3000 rpm and filtered again. The pooled extracts were scintillation counted and the non-extractable residues were determined in the sediments after drying as mentioned under 3.7.4. During the application of this extraction technique, the mass balance was set up after every extraction step.

3.7.7 Solid phase extraction

Method description

For the RTLC screening of the water samples, diclofenac and its transformation products had to be concentrated. For this purpose, solid phase extraction (SPE) was used as a clean up and enrichment step using strata-X cartridge (Strata^{TM}-X 33u µm cartridge, polymeric sorbent, reversed phase, Phenomenex, Aschaffenburg, Germany). Prior loading of water samples, the cartridges were conditioned with 5 mL of acetone, 5 mL of methanol, and 5 mL of demineralized water (pH 2). Afterwards, the aqueous phase was adjusted at pH 2.5 with diluted HCl, to prevent diclofenac from taking its ionic form [Kot-Wasik et al., 2006]. After loading the samples, at a flow rate of 3 mL/min, the cartridges were dried under gentle stream of nitrogen. Afterwards, diclofenac and its transformation products were eluted with 5 x 1 mL methanol. The radioactivities in 2 aliquots (50 and 100 µL) of the methanol eluent, after mixing with 10 mL Quicksafe N (Zinsser, Frankfurt, Germany), were measured by LSC to ensure complete elution of the radioactivity. Additionally, the radioactivities in 2 aliquots of the percolates were measured by LSC for determination of break through of the diclofenac and its transformation products. Finally, the methanol eluent was stored in brown glass vials at 4 ºC until RTLC analysis and the cartridges were stored at - 20 ºC until the setup of the mass balances. For quality assurance, the extraction efficiency of Strata-X material was preliminarily tested by spiking of ^{14}C-diclofenac in 50 mL water samples at a radioactivity level of 0.14 MBq/L each. The water was then extracted as mentoned above. The recoveries were found to be 93 % ± 3 **[Appendix, Table A1]**. Additionally, the difference between the radioactivity determined in the water phase and in the percolate were determined for

calculation of losses occurred during the SPE. The radioactivity % in the eluent was calculated according to the following equation:

Eluent = A1 x V2 x 100/A2 x V1

where:

A1: Radioactivity in the analyzed aliquot (Bq).

A2: Initially applied radioactivity (Bq).

V1: Volume of the analyzed aliquot (mL).

V2: 5 mL.

100: Conversion factor to percent.

Strata-X sorbent material

Since the concentrations of ^{14}C-diclofenac in the water phases are relatively low, enrichment steps are needed especially when it occurs together with its transformation products which are expected at lower concentration than ^{14}C-diclofenac. Although several extraction methods have been published, SPE remains the most widely used method for extraction and clean up of diclofenac from water samples either alone or in combination with the other methods [Ahrer et al., 2001, Bartels and von Tuempling Jr, 2007, Antonic and Heath, 2007]. In the literature, different types of SPE cartridges were reported to be suitable for extraction of environmental samples containing diclofenac and/or it transformation products such as Strata-X [Hilton et al., 2003, Kosjek et al., 2005, Drover and Bottaro, 2008], Oasis HLB [Tixier et al., 2003, Bueno et al., 2007, Chefetz et al., 2008, Chen et al., 2008, Kosjek et al., 2008, Terzic et al., 2008, Bui et al., 2009], BakerBond PolarPlus [Stuelten et al., 2008a, b], Isolute ENV+ [Groning et al., 2007] and Oasis MAX in combination with Oasis HLB [Agueera et al., 2005, Bartels and von Tümpling Jr, 2007]. In the present study, the polymeric sorbents, Strata-X (polydivinylbenzene resin containing 2-pyrrolidinone groups) was selected for extraction of diclofenac and its transformation products from water samples due to its sorbent material which improve the retention of acidic, basic and neutral compounds. This may be attributed to its modified polymeric surface of this material that has hydrophilic, lipophilic, and π-π retention interaction mechanisms **[Figure 12].** Therefore, Strata-X has been used simultaneously in the analysis of multi-class pharmaceuticals in water samples [Hilton and Thomas, 2003, Roberts and Thomas, 2006].

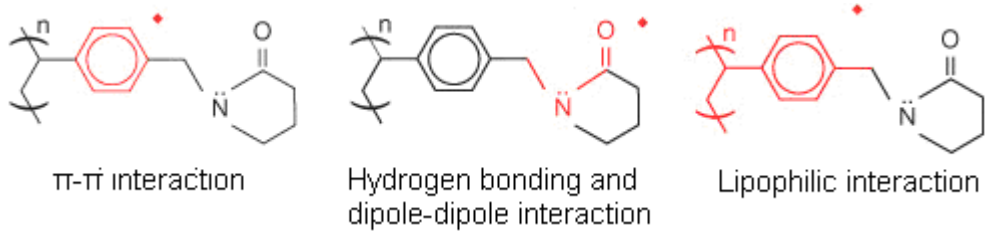


Figure 12: Structure of Strata-X sorbent material showing different retention mechanisms

3.7.8 Screening of ^{14}C-diclofenac and its transformation products

Method description

In all extracts, the composition of the radioactivity was investigated using radio thin layer chromatography (RTLC) either Tracemaster 20 Automatic TLC-linear analyzer B 284, (Berthold, Muenchen, Germany) or the Rita Star TLC-Scanner (Ray Test, Straubenhardt, Germany). The substances were separated on silica gel plates, 60 F_{254}, 20 x 20 cm^2 (Merck, Darmstadt, Germany). The plates were divided into several tracks, one of which was reserved for ^{14}C-diclofenac reference standard for R_f comparison purpose. Extracts and standards were spotted by means of disposal micro-pipettes in the pre-concentration zone (2 x 20 cm^2) from each other and from the edges of the plate. The volumes applied onto the plates were depended on the radioactivity amounts of the analytical solutions. After the evaporation of the solvents in a fume hood, the plates were placed into the development chamber filled with approximately 100 mL of the developing solvent. Using normal phase TLC plates, two different solvent systems were used for detection of diclofenac and its transformation products. First solvent consisted of n-hexane/ethyl acetate/acetic acid (50:50:1 v/v/v) while the second was dichloromethane/methanol/25 % ammonia (85:14:1 v/v/v). For comparison purposes, the extracts of some irradiation experiments were additionally developed on reversed phase (RP-18) silica gel plates (Merck, Darmstadt, Germany), 60 F_{254s} (20 x 20 cm^2) using acetonitrile/water/formic acid (90:10:0.02). This ratio was preliminary determined after testing different solvent ratios starting from (50:50:0.02). All plates were developed to a distance of 18.5 cm in a closed development chamber and were then dried in a fume hood. Then, the TLC plates were scanned for certain time, according to the radioactivity amount in each spot, at an amplifier voltage of 900 V. A mixture of argon and methane (90:10) was used as a purge gas. The software Chroma 2D or Gina star were used to evaluate the autoradiographic data and the peaks were integrated manually after smoothing. The percentage of diclofenac detected as a parent compound was calculated from the following equation:

Substance (%) = A x ROI/100

where:

A: Radioactivity percentage in the applied extract.

ROI: Percentage of region of interested (computed by the soft ware after manual integration).

100: Conversion factor to percent.

For quality assurance, the radiochemical purity of ^{14}C-diclofenac was checked before starting each set of experiments.

Principles of RTLC

In contrast to LSC, RTLC is designed to detect alpha and beta radiation emitted by radioactive substances which exist in the solid state. These substances were previously spotted as analytical solutions onto TLC plates and left for drying. The measurement is occurred by means of a sensitive movable proportional counter tube (detector). Additionally, RTLC instrument detects not only presence of, for example, beta particles and measure the resulting pulse, but also determine the position or the distribution of separated radioactive substances on a developed TLC plate. RTLC mainly comprises of ionization tube in which a positively charged counting wire (anode) and a ground wire (cathode) are involved. For amplification of the signals, a sufficiently high voltage is applied between the electrodes (900-1800 V). Additionally, insulating counting gas consisting of 10 % methane and 90 % argon is allowed to flow constantly through the tube as a purge gas. This mixture is preferred because it is nearly non-flammable and allows the counter to operate at lower voltage than with methane alone (3000 V). In the standby mode, the potential difference between the electrodes is considered to be constant and hence stable base line is obtained. On the other hand, when a ^{14}C-radioactive substance, present on a TLC plate, is placed parallel to the counter tube, the gas molecules are bombarded with beta particles leading to ionization of their atoms. As a result, free electrons and positive ions are formed. This process is known as a primary ionization process. According to their polarity, the charged particles are accelerated towards the anode and the cathode. In their way towards the anode, the electrons move with increasing speed and gain more and more energy. These electrons cause further ionization to the other gas atoms leading to formation of further electrons and positive ions. This process is known as a secondary ionization process. The latter continuous resulting in generation of charge cascade (charge multiplication process) from the point of primary ionization to the anode wire. The generated charge is proportional to the ionization energy of the emitted beta particles. Additionally, the charge existed in the anode wire generates an induced charge in the cathode which is subsequently discharged in two

directions causing two different delay times in the normal current. The time is then passed through a time to digital converter and the difference between the digits determines the start and the stop margin (position) of the signal on the TLC plate. Afterwards, the R_f value of a certain radioactive substance is automatically determined. Furthermore, the intensity of radioactivity is given in impulses without referring to the time unit, because the time of the measurement depends on the amount of radioactivity spotted on the plate. The identification of substances is made through the comparison of their R_f values with reference standards developed on the same plate.

3.8 GC/MS experiments

3.8.1 Detection of diclofenac after derivatization

Due to low vapor pressures and polarity, the carboxylic group of diclofenac should be converted to the ester derivate in order to improve its volatility and the thermal stability for more sensitive GC/MS detection [El Haj et al., 1999, Antonic and Heath, 2007]. Although diazomethane was frequently used in derivatization of diclofenac, it was excluded in the present study due to its high toxicity and low stability. Alternatively, pentafluorobenzyl bromide (PFBBr; obtained from Sigma-Aldrich, Deisenhofen, Germany) was used. The derivatization process was applied according to Moeder et al. (2007). After addition of 50 µL of 10 % anhydrous K_2CO_3 to 1 mL diclofenac standard in acetone (15 ng/µL), 20 µL of 5 % PFBBr were added and the reaction mixture was heated for 4 h at 60 °C. After cooling, the reaction mixture was evaporated up to dryness. The residues containing diclofenac pentafluorobenzyl ester were then dissolved in 5 mL of n-hexane. The excess of the reagent was removed by addition of 10 mL of pure water and shaken at least for 1 min. Afterwards, the organic layer was separated, dried with anhydrous sodium sulphate, decanted, reduced to 1 mL and stored at -20 °C until GC/MS analysis.

3.8.2 Detection of diclofenac without derivatization

Different concentrations (1-10 ng/µL) of diclofenac standard solutions were prepared in acetone or ethyl acetate and then directly injected into GC/MS to check the thermal behavior of diclofenac.

3.8.3 Detection of phototransformation products of diclofenac

In order to determine the structure of the phototransformation products of diclofenac in water, 500 mL of pure water were placed in the sample container of the irradiation apparatus and fortified with 5 mL of aqueous diclofenac standard solution (10 ng/µL). The container was then incubated under the irradiation apparatus at 20 ± 2 °C for 5 h. A-100 mL water sample was taken every hour, liquid-liquid extracted (cf. 3.8.4) and then analyzed for diclofenac and

its phototransformation products. The same procedure was repeated for three days (10 light/14 dark) using 300 mL pure water and 3 mL of aqueous diclofenac standard solution (10 ng/µL). A 100-mL water sample was taken every day and analyzed before and after derivatization, by means of GC/MS. The samples were collected in such intervals because the transformation rate of diclofenac was unknown.

3.8.4 Liquid-liquid extraction

After transferring water samples into a separating funnels, diclofenac and its phototransformation products were extracted five times with 30 mL ethyl acetate each. Prior extraction, 2 g sodium chloride were added to improve phase separation. Subsequently, the extracts were pooled. Samples analyzed by GC/MS were filtrated and dried over 25 g anhydrous sodium sulphate [Bartels and von Tuempling Jr., 2007]. The filtrated extracts were evaporated to 1 mL. The extracts were then stored in brown glass vials and stored at -20 °C until analysis.

For quality assurance, the liquid-liquid extraction procedure was preliminarily tested by different techniques. For radiotracer analysis, ^{14}C-diclofenac was spiked in 100 mL water at a radioactivity level of 0.35 MBq/L. For GC/MS and LC/MS/MS, 100 mL water samples were fortified with diclofenac at the concentration level of 0.1 mg/L. For HPLC/UVD, 100 mL water samples were spiked at the concentration level of 1 mg/L. The recoveries calculated after radiotracer, GC/MS, HPLC/UVD and LC/MS/MS analysis were 94 ± 2 %, 89 ± 1 %, 92 ± 2 % and 89 ± 1 %, respectively **[Appendix, Table A2-A5]**.

3.8.5 GC/MS Instrumentation

GC/MS operation system

GC/MS determination was performed using a Shimadzu (GC-17A) gas chromatograph equipped with Shimadzu AOC-20i programmable auto injector. The gas chromatograph was coupled to a Shimadzu QP 5050A (Shimadzu, Kyoto, Japan) mass selective detector (MSD). The ionization was done in electron impact mode (EI) with electron energy of 70 eV. In case of identification purposes, the mass spectrometer was operated in full scan mode at the mass of range 60 - 600 amu and, in case of quantitation of diclofenac and diclofenac pentafluorobenzyl ester it was operated in a single ion monitoring mode (SIM). The 1-µL aliquots were injected into the split/splitless injector port (250-280 °C). A DB-5 MS fused silica capillary column with 30 m length, 0.25 µm film thickness and 0.25 mm inner diameter was used (J&W Scientific, Folsom, CA, USA). The GC oven temperature program started at 60 °C, held for 2 min and was then increased to 300 °C with a rate of 10 °C/min. This final

temperature was held for 20 min. The transfer line temperature was maintained at 300 °C and the carrier gas flow rate of helium was 1 mL/min.

GC/MS principles

GC/MS is an instrument that combines the advantages of gas chromatography and mass spectrometry. It is a powerful widely used tool for the separation, identification and/or quantitation of a mixture of substances. GC/MS is generally consists of an injection port, column and a MS detector. It is designed to measure the substances when they are in the gas phase. Therefore, a solvent containing mixture of substances is introduced, by means of micro syringe, to a very hot chamber known as the injection port. As a result, flash evaporation occurs transferring the substances from the liquid into the gas phase. During this process, however, the substances undergo thermal stress. Hence, GC is preferably used for the analysis of less polar and thermal stable analytes. Afterwards, by means of inert carrier gas (mobile phase) such as helium, the vaporized substances are introduced to a very long, narrow and temperature controlled column known as the capillary column. The inner wall of the column is covered with a thin film known as the stationary phase. According to their polarity and boiling point, the substances are separated depending on their affinity to the mobile or the stationary phase. As the separated substances emerge from the column opening, they enter into the MS unit through the transfer line. There, the substance is ionized and the generated ions are separated according to their mass to charge ratio. Among ionization techniques, electron impact ionization is considered the most common and perhaps the standard form of ionization. The large amount of structural information is yielded by the full scan mass spectra obtained under electron impact (EI) ionization and the possibility of using commercial libraries, making screening and identification of unknowns feasible, are the most two important advantages of GC/MS technique.

3.9 HPLC experiments

3.9.1 ^{14}C-diclofenac irradiation tests

Because the irradiation period mentioned under 3.7.2 was found to be too long to detect any phototransformation products of diclofenac, additional irradiation experiments were conducted to get an idea about their nature. Additionally, labeled and unlabeled diclofenac were mixed together in order to enhance the concentration of labeled diclofenac. Therefore, irradiated samples can be also analyzed by means of HPLC. For this purpose, 500 mL of pure water was fortified with 500 µL unlabeled aqueous diclofenac standard solution (1 µg/µL) and 150 µL of labeled diclofenac (925 Bq/µL) in the sample container of the irradiation apparatus. The container was then fixed tightly into the irradiation apparatus and kept at 20 ± 2 °C for different irradiation intervals (1-5 h).

During the incubation time, a 100-mL water sample was removed every hour for HPLC/UVD and radiotracer analysis. Afterwards, the water phases were liquid-liquid extracted by means of ethyl acetate (cf. 3.8.4). For the setup of mass balances, the radioactivity was then measured in the water phases remained after liquid-liquid extraction and in the ethyl acetate extracts before and after rotary evaporation. Additionally, the evaporated extracts were screened for diclofenac and its transformation products (cf. 3.7.8). The partitioning behavior for diclofenac and its phototransformation products were indicated by the determination of the extraction efficiency of ethyl acetate for the radiotracer residue after each irradiation interval. For HPLC analysis, the ethyl acetate extracts were reconstituted in 1 mL methanol to be suitable for the HPLC system. For quality assurance, the solvent exchange step from ethyl acetate to methanol was preliminary tested by means of HPLC/UVD. The recoveries were found to be 100 ± 2 % **[Appendix, Table A6]**. As a second extraction step, the water remained after liquid-liquid extraction process was subjected to SPE (cf. 3.7.7). Afterwards, the eluted analytes were screened by the HPLC for presence of phototransformation products of higher polarity that could not be extracted by means of ethyl acetate.

3.9.2 Unlabeled diclofenac irradiation tests

The unlabeled test was performed almost the same as the radiotracer test (cf. 3.9.1). In contrast, the whole irradiation time was prolonged to be 10 h instead of 5 h aiming to transform diclofenac completely, sampling was performed every 2 h instead of 1 h.

3.9.3 HPLC Instrumentation

The HPLC analysis was performed using Hewlett Packard 1050 Series HPLC-system (Hewlett Packard, Waldbronn, Germany) which consisted of injector, pump and variable wavelength UV detector. Diclofenac and its phototransformation products were separated in a 250 mm x 4 mm LIChrospher® 100 RP-18 column (5 µm) (Agilent Technologies GmbH; Waldbronn, Germany). As a confirmative step, two different isocratic mobile phases, degassed by helium, were used. The first consisted of methanol/water/phosphoric acid (70:30:0.1 v/v/v) and the second consisted of acetonitrile/water/formic acid (50:50:0.1 v/v/v). The flow rate was 1 mL/min. An injection volume of 25 µL was used for all samples. Column temperature was 28 °C. The quantitation of diclofenac was performed at λ = 280 nm, while the phototransformation products were detected at λ = 254 nm.

Later on, the absorption spectrum of diclofenac and its phototransformation products were obtained using HPLC/DAD. For this purpose, an Agilent 1100 series HPLC-system (Agilent

Technologies, Waldbronn, Germany) equipped with a binary pump, column thermostat, thermostated autosampler and DAD detector was used. The mobile phase consisted of acetonitrile/water/formic acid (50:50:0.1 v/v/v) was used at a constant flow of 1.0 mL/min. The previously mentioned column was kept at a constant temperature of 20 °C. The volume of injection was adjusted at 20 µL and the vials were kept at 10 °C in the autosampler. The absorption spectra were recorded in a wavelength ranging from 200 to 400 nm.

HPLC principles

High performance liquid chromatography (HPLC) is an analytical technique used for separation of mixture, identification and/or quantitation of organic and inorganic compounds. The instrument comprises of mobile phase reservoirs, pump, injector loop or autosampler, sample valve, column, detector and data analysis system. Analytes to be analyzed should be dissolved in HPLC compatible solvents. By means of the injector, a small volume of this solvent is introduced to the stream of mobile phase. The latter is forced under high pressure by the action of the pump into the stationary phase of the column. As they travel through the column, the analytes are interacted with the stationary phase and separated according to their number of interactions or their affinity for partitioning between the mobile and the stationary phase. Afterwards, the separated analytes reach to the detector to be recorded at certain time known as retention time. The retention time under particular conditions is considered a unique identifying characteristic of a given analyte.

3.10 LC/MS/MS experiments

3.10.1 Pure water tests

For structural identification of phototransformation products of diclofenac an additional irradiation experiment was preformed in which the samples were prepared exactly as mentioned under 3.9.2 with an exception that they were diluted before analysis by LC/MS water in ratios of 50:50 (v/v).

3.10.2 Water/sediment tests

Irradiation experiments

For structural elucidation of phototransformation products of diclofenac in water/sediment systems, an additional irradiation experiment was preformed in which the system was equilibrated and, extracted as mentioned under 3.6.1 with the following exceptions. Only one sediment type was used (S3), the fortification level of diclofenac was 500 µL (1 µg/µL), the irradiation period was 5 h instead of 3 days, the water phase was extracted by means of liquid-liquid extraction instead of SPE, and the ^{14}C-carbon dioxide trap was not analyzed for determination of mineralization rate. The other exception was the handling of the ethyl

acetate extracts of both water and sediment compartments. These extracts have to be carefully cleaned before LC/MS/MS analysis. For this purpose, the extracts of water and sediment samples were evaporated to 1 mL, then dried under a gentle nitrogen stream and reconstituted in a final volume of 5 mL by adding ethyl acetate/cyclohexane (50:50 v/v). If necessary, the extracts were then stored at - 20 °C until the clean up steps.

Dark experiments

For structural elucidation of biotransformation products of diclofenac in water/sediment systems, another dark experiment was conducted by the same way as mentioned for the irradiation experiments with only one exception that the flasks were stoppered and incubated in the dark at 20 ± 2 ºC for 7 days. The air above the water phase was exchanged after 4 days to maintain aerobic conditions.

3.10.3 GPC clean up

The Gilson GPC unit (Middleton, UAS) consisted of isocratic HPLC-pump model 302, auto-sampler injector model 231, dilutor model 401, fraction collector model 201, and glass chromatographic column filled with 38-39 cm of bio-beads S-X8 (dimensions: 59 cm length, 2.9 cm inner diameter). The eluent was a mixture of ethyl acetate and cyclohexane (50:50 % v/v). The flow rate was set at 5.0 mL/min and the injection volume was 4.0 mL. The elution was programmed as 23 min waste, then 12 min analyte fraction containing diclofenac and its phototransformation products, and finally 10 min rinsing. Prior GPC clean up, the extract was micro-filtered using PTFE syringe filter. The 4-mL aliquots were injected into the GPC apparatus. The fractions were separated as 0-115 mL, 115-175 mL and 175-225 mL for the forerun, analyte, and tailing fractions, respectively. The analyte fractions were rotary evaporated to 1 mL then to dryness under a gentle nitrogen stream. Afterwards, the residues were recomposed to a final volume of 2 mL in methanol. The extracts were stored in amber vials and stored at - 20 °C until chromatographic analyses. Before LC/MS/MS analysis, the extracts were diluted 10 times with LC/MS water/methanol (50:50 v/v) to avoid saturation of the quadrupole and to meat the calibration curve. For quality assurance, the clean up procedure was preliminary tested by means of HPLC/UVD. The recoveries were found to be 97 ± 5 % **[Appendix, Table A7]**.

3.10.4 Alumina column clean up

Preliminary, a second clean up step was tested aiming to get cleaner sediment extracts. For this reason, another irradiation experiment was conducted and after the GPC clean up step, the extracts were still coloured green. Therefore, alumina column for clean up of polar compounds was tried according to Batarseh (2003). A chromatographic column (2 cm i.d. x

30 cm) was plugged with a piece of glass wool, and packed with 10 g alumina (90 neutral, 70-230 mesh; Merck, Darmstadt, Germany) previously activated at 220 °C over night, deactivated with 12.5 % water and shaken for at least 4 hours using a horizontal shaker. The sediment extracts were transferred to the alumina column and then eluted with 5 mL ethyl acetate followed by 100 mL of methanol/ammonia mixture (99:1 v/v). The eluent was then rotary evaporated to 2 mL and then analyzed.

3.10.5 LC/MS/MS Instrumentation

LC/MS/MS principles

The identification and structural elucidation of unknowns are a challenging task. Therefore, LC/MS/MS/ESI QTRAP was selected for identification of the phototransformation products of diclofenac. It mainly comprises of HPLC, ion source, mass analyzer and detector **[Figure 13]**.

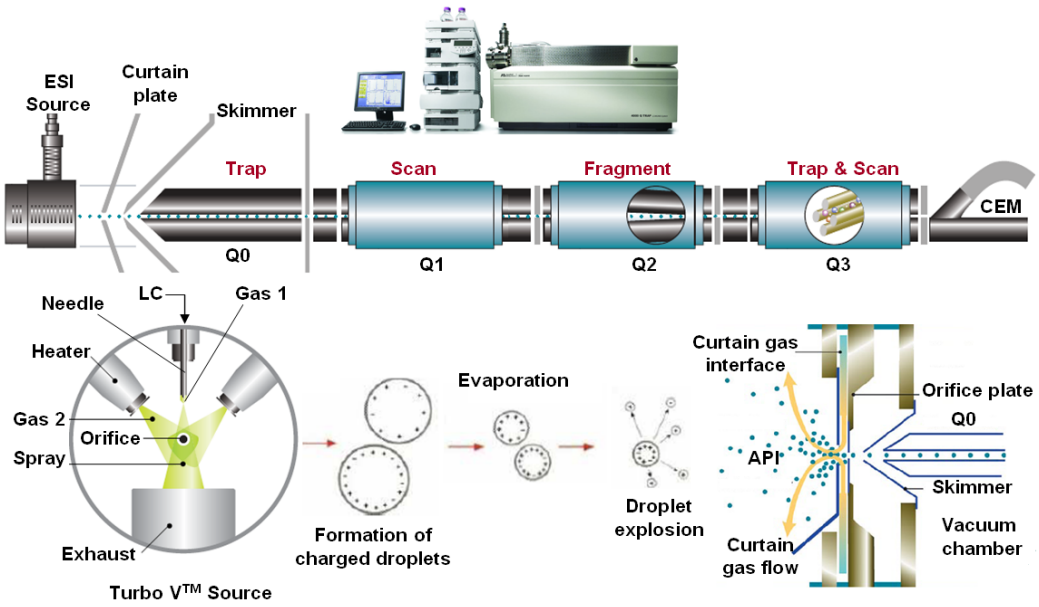


Figure 13: LC/MS/MS/ESI QTRAP (AB Sciex, Ontario, Canada) instrument showing the mechanism by which ions are formed in the Turbo V™ ESI ion source before entring the MS/MS region

Ion source

Among the atmospheric pressure ionization (API) techniques, in which the formation of ions occur at the atmospheric pressure, electrospray ionization (ESI) and atmospheric chemical ionization (APCI) are considered the most commonly used in LC/MS/MS analyses. Both are sensitive and soft ionization modes that produce positive or negative ions from polar substances introduced as a stream of the HPLC eluent through a needle. In case of ESI, the

ionization occurs in the liquid phase by the application of high electric fields to the needle. From the liquid phase, spray of charged droplets is then formed by the aid of a nebulizer gas. As the solvent evaporates by means of a vaporizer gas, the droplets shrink and the repulsion forces between the charged ions increase. Subsequently, the droplets become unstable and hence dissociate into smaller droplets. This process is repeated several times resulting in separation of the ions from the surface (due to the high repulsion force) or explosion of the droplets. In both cases, gas phase ions are produced. In case of APCI, gas phase molecules are produced by means of heater at the LC/MS/MS interface. Afterwards, the chemical ionization occurs in plasma created by corona needle discharge. Due to the volatilization before ionization, APCI is limited to smaller molecules than ESI which is more sensitive, softer, and applicable for a wide range of substances especially the thermolabile ones. However, APCI has the advantage of being less influenced by sample matrix effects. Alternatively, atmospheric pressure photoionization in which ionization occurs by a xenon lamp instead of a corona discharge needle is considered the third type of API but less applicable.

Mass Analyzer

The vacuum region of the mass spectrometer is separated from the spray by a capillary orifice. Via this orifice **[Figure 13]**, precursor ions enter into Q_0 region in presence of the curtain gas which is responsible for the declustering of the ions and protection of the curtain plate from striking by these ions. At Q_0 region, fragmentation can be induced and controlled by the declustering potential. Additionally, ions can be accumulated in Q_0 region in order to enhance their concentration before entring Q_1 region to be scanned. Afterwards, ions can be introduced to Q_2 region (collision cell). There, it can be additionally fragmented by means of an inert gas (collision gas) in order to produce characteristic fragment ions (product ions). The degree of fragmentation is directly proportional to the pressure of the collision gas and the collision energy. After fragmentation, product ions enter Q_3 region to be scanned. Before scanning, Q_3 can optionally be used as a linear ion trap (LIT) mass spectrometer in order to enhance the sensitivity by the trapping of ions.

In the literature, several advantages have been reported for LC/MS/MS to be widely accepted as a main tool in the structural elucidation purposes [Abdel-Hamid et al., 2001, Ahrer et al. 2001, Jansen et al., 2005, Fatta et al., 2007, Chen et al., 2008]:

1- In contrast to GC/MS, it has high capability for analyzing very polar, less volatile, thermolabile compounds in shorter run time without a need for derivatization using highly toxic and carcinogenic derivatization reagents such as diazomethane.

2- In comparison with single MS, tandem MS provides more selectivity and sensitivity in complicated matrices because it is associated with higher and more stable signal to noise ratios as a result of the specific fragmentations of the isolated precursor ions and the elimination selectivity of the background noise. Additionally, complete chromatographic resolution of analytes, with the same molecular mass, is not a real problem as long as the overlapping analytes have different product ions.

3- Hybrid QTRAP mass analyzers combine both high selective triple quadrupole (QqQ) scan functions and high sensitive LIT scan functions in a single instrument. In the past, a sample had to be analyzed with two different instruments (QqQ and a 3D ion trap) to obtain similar information. Additionally, the trapping capacity and the ion path length of the LIT systems are 70 and 30 times, respectively, higher than the 3D systems leading to much higher sensitivity.

4- Compared to LC/TOF/MS, QqQ is more applicable in quantitative purposes as it provides a higher effective linear dynamic range.

5- Several scans can be performed in the QqQ mode such as:

   a) Q1 or Q3 scan: A single full MS scan in which the first (Q1) or the third (Q3) quadrupole search in a particular mass range for all ions.

   b) Product ion scan (PI): An MS/MS full scan in which the Q1 is fixed to transmit a certain precursor (mother) ion and, after fragmentation in Q2, the Q3 searches in a particular mass range for all product (daughter) ions generated by the precursor ion.

   c) Precursor ion scan (Prec): An MS/MS full scan in which the Q3 is fixed to detect a certain product ion after fragmentation in Q2, and the Q1 searches in a particular mass range for all precursor ions generating the specified product ion.

   d) Multiple reactions monitoring (MRM) scan: An MS/MS full scan in which Q1 is fixed to transmit a certain precursor ion and, after fragmentation in Q2, Q3 is fixed to detect a certain product ion generated from the precursor ion.

6- LIT offers some extra scans such as:

   a) Enhanced MS (EMS): A single full MS scan in which ions are transferred directly to Q3, trapped for a certain time to enhance the concentration and finally scanned in a particular mass range for all ions.

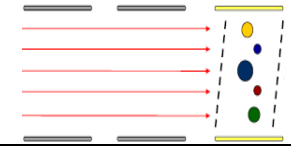
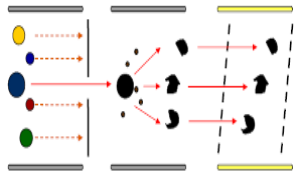
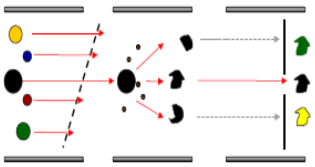
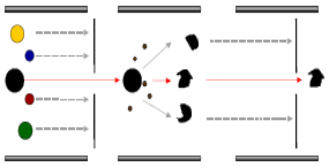
   b) Enhanced product ion scan (EPI): An MS/MS full scan in which the Q1 is fixed to transmit a certain precursor ion and, after fragmentation in Q2, the product ions are trapped for a certain time in Q3 to enhance the concentration and finally scanned in a particular mass range for all ions generated by the precursor ion.

7- Presence of the built-in information dependent acquisition (IDA) software allows combinations of two different scans, i.e. EMS and EPI, in the same run providing

maximum information by fewest numbers of injections. Hence, rapid identification and confirmation of unknowns could be obtained. In the IDA, the first and the second scan are known as survey and dependent scan, respectively. The instrument is allowed to switch to the dependent scan when the predefined criteria (such as ion intensities or threshold) are acquired during the survey scan. Finally, after a short time, the instrument is switched back to the initial conditions.

The selected scan modes used in the present study and their applications are shown in **Table 6.**

Table 6: Scan modes selected for the present study

<table>
<tr><th></th><th>Q1 function</th><th>Q2 function</th><th>Q3 function</th><th>Scan type</th><th>Application</th></tr>
<tr><td>  </td><td>------</td><td>------</td><td>Trap&Scan all ions</td><td>EMS</td><td>Confirm presence of known ions</td></tr>
<tr><td rowspan="2">  </td><td>Fixed ion</td><td rowspan="2">Fragmen-tation</td><td>Trap&Scan all ions</td><td>EPI</td><td rowspan="2">Identification of fragments</td></tr>
<tr><td>Unknown ion</td><td>IDA</td><td>EMS-EPI</td></tr>
<tr><td>  </td><td>Scan all ions</td><td>Fragmen-tation</td><td>Fixed ion</td><td>Prec</td><td>Identification of precursors</td></tr>
<tr><td>  </td><td>Fixed ion</td><td>Fragmen-tation</td><td>Fixed ion</td><td>MRM</td><td>Quantifcation of analytes</td></tr>
</table>

On the other hand some disadvantages have been reported for LC/MS/MS [King et al., 2000, Buchberger, 2007]

1- In the form of signal suppression, the ionization modes used in LC/MS/MS such as ESI are more influenced by the matrix components, eluting at the same time with the analytes, than the ionization modes used in GC/MS such as EI or CI. However, this problem was avoided in the present study by means of dilution. Gomez et al. (2006) reported signal suppression of 62 % and 7 % for diclofenac in spiked hospital effluent wastewater before and after dilution (1:2), respectively.

2- The mass accuracy of QqQ is lower than TOF mass analyzer.
3- In comparison to 3D, multiple ion transitions MS^n is not possible in case of QTRAP mass analyzer.

Method description

The 4000 QTRAP LC/MS/MS system consisted of an Agilent 1200 series liquid chromatograph including a vacuum degasser, a binary pump, and an high performance autosampler (Agilent Technologies, Waldbronn, Germany) coupled to an Applied Biosystems/MSD SCIEX 4000 QTRAP tandem mass spectrometer equipped with an electrospray ionization interface, Turbo V^{TM} source, (Applied Biosystems GmbH, Darmstadt, Germany). The LC/MS/MS system was controlled by Analyst software (version 1.4.2 Applied Biosystems) with a built-in information depending acquisition scan function.

The electrospray ionization interface operated in negative ion mode $[M-H]^-$. Diclofenac and its phototransformation products were separated on a Gemini 110A C_{18} reversed-phase column (150 mm × 3 mm i.d., 5 µm particle size; Phenomenex, Aschaffenburg, Germany). Acetonitrile/0.5 % formic acid (aq.) [50:50 (v/v)] was used as the mobile phase in the isocratic mode. The flow rate was 0.3 mL/min. The injection volume was 20 µL.

The compound dependent (ion optics) parameters were automatically optimized by infusion method (continuous injection of diclofenac standard solution into the MS/MS directly) using an external pump. The optimal values for the declustering potential (DP), entrance potential (EP), collision cell energy (CE), and collision cell exit potential (CXP) were - 40, - 10, - 16, and - 11 V, respectively. The collision affected dissociation gas (CAD) was adjusted at high pressure. On the other hand, the ion source parameters were automatically optimized by flow injection analysis (FIA) method. Nitrogen was used as curtain gas (CUR), nebulizer gas (Gas1), vaporizer or auxiliary gas (Gas 2), at 20.7 x 10^4, 34.5 x 10^4, 34.5 x 10^4 Pa, respectively. The ion spray voltage (IS) was - 4 kV. The interface heater (ihe) was 100 °C and the turbo gas temperature (TEM) was optimized manually at vaporization temperature of 200 °C.

The parameters were optimized using diclofenac standard solution for $[M-H]^-$ (294 m/z) since the other phototransformation products are not available as standard solutions. The identification of the unknown phototransformation products of diclofenac was carried out using information depending acquisition (IDA) experiments which allow combination of two different scan types in the same run. Therefore, enhanced full mass scan (EMS) was used

as a survey scan (mass range m/z 100–500, scan time 0.4 s, scan rate 1000 amu/s, trap time 20 ms) and the enhanced product ion scan (EPI) was used as dependent scan. The IDA was established to record the most abundant product ion of the ions that exceed 15 x 10^4 counts in the scan range of m/z 100–500. Precursor ion scan (Prec) was applied after that for identification of the precursor ions of m/z 250, 214, 196 and 185 (mass range m/z 150–400, scan time 3 s, scan rate 83 amu/s). Quantitation for diclofenac was performed by multiple reaction monitoring mode (MRM) for the deprotonated precursor ion (m/z 294) and its related product ion (m/z 250) using the external standard method. Quadrupoles Q1 and Q3 were set at unit resolution.

For quality assurance, blanks were carried out to check for interference and contamination during the GC/MS, HPLC and LC/MS/MS experiments. Most of the samples were prepared in duplicate and the measurements were performed two times. Linear regression analysis was used in the quantitation of the diclofenac. The quantitation was based on direct peak area measurements that were automatically computed using external calibration standards. The external calibration curves were prepared by plotting the total peak areas of diclofenac versus the concentrations. The linearity was accepted when the regression coefficients were ≥ 0.995 and the slopes were consistent with the measured peak areas. The complete calibration curve parameters are listed in **Appendix, Table A8.** However, due to the lack of authentic reference standards, concentrations of transformation products were expressed as peak areas. Additionally, the stability of the diclofenac under the storage conditions was continuously checked by comparing previously used calibration series with freshly prepared ones.

3.11 NMR experiments

For structural confirmation and quantitation purposes, large amount of the most abundant phototransformation product of diclofenac, namely 8-chloro-9H-carbazole-1-acetic acid, was tried to be obtained in pure form through chromatographic isolation.

3.11.1 Sample preparation

Diclofenac (1 g) was dissolved in 200 mL water and then irradiated for 5 h. Longer irradiation time was avoided because phototransformation products may be as photosensitive as diclofenac. The water phase was then liquid-liquid extracted as mentioned before using ethyl acetate 20 x 50 mL. Afterwards, the extract was rotary evaporated to dryness at 40 °C and the residues were then dissolved in 20 mL methanol. Preliminary, the effect of irradiation

time and the amount of diclofenac initially applied on the transformation processes were tested.

3.11.2 Isolation by column chromatography

For fractionation, the methanolic solution was transferred to a preparative reversed-phase chromatographic column (35 cm length, 4 cm inner diameter, Lichroprep C_{18}, particle size 40-63 µm; Merck, Darmstadt, Germany). Acetonitrile/water/formic acid (50:50:0.1 v/v/v) was used as a mobile phase under low pressure. Previously, the column was conditioned with 1 L of the same mobile phase. The eluent was then collected in several fractions: 2 x 75 mL flasks and 70 x 20 mL tubes. During fractionation, rapid tests by means of reversed -phase thin layer chromatography were carried out to check for the separation capacity. Fractions containing mainly the target compound were combined, rotary evaporated to dryness under reduced pressure at 40 °C and the residues were then dissolved in 5 mL methanol for additional purification.

3.11.3 Purification by semi-preparative HPLC

For further separation, a semi-preparative HPLC (Merck, Darmstadt, Germany) was used. It consists a of Merck-Hitachi L-6200 intelligent pump equipped with Merck-Hitachi L-4200 UV/VIS detector set to λ = 254 nm, 0.1 mL injection loop and Luna C18 column (250 mm x 10 mm, 5 µm particles; Phenomenex, Aschaffenburg, Germany). The solvent system was A: water with 0.1 % formic acid and B: acetonitrile/water/formic acid (50:50:0.1, v/v/v). The gradient was programmed as shown in **Table 7**. The flow rate was 4.6 mL/min. Within the 5-min post-run period, the initial conditions were adjusted again. A 0.1-mL volume of the last methanolic solution was injected successively into the semi-preparative HPLC system. Phototransformation product 1 was collected manually during numerous runs. The purity of the isolated phototransformation product 1 solutions was determined by analytical HPLC as described before. After removal of the pooled mobile phase by rotary evaporation under reduced pressure at 40 °C, the residue was dissolved in deuterated methanol (CD_3OD) for ^{1}H- and ^{13}C-NMR analysis.

Table 7: HPLC gradient for purification of 8-chloro-9H-carbazole-1-acetic acid

Time (min)	A % Acetonitrile/water/formic acid (50:50:0.1, v/v/v)	B % 0.1 formic acid aqu.	C% Acetonitrile
0	40	60	0
20	100	0	0
30	100	0	0
30.1	0	0	100
35	0	0	100
40	40	60	0

3.11.4 NMR instrumentation

NMR spectra were recorded by means of a Bruker Avance II 600 spectrometer equipped with a CP-TCI-z cryoprobe (Bruker BioSpin, Rheinstetten, Germany) at 600 MHz (^{1}H) and 150 MHz (^{13}C). The solvent used was deuterated methanol (CD_3OD). Internal chemical shift references were tetramethylsilane (TMS) for the ^{1}H spectra (δ = 0.00 ppm) and the solvent peak for the ^{13}C spectra (δ = 77.01 ppm). Two-dimensional H,C-hsqc and H,C-hmbc spectra were recorded using standard Bruker pulse programs. Sweep widths and pulse delays were optimized for the sample under investigation. The ^{1}H-NMR spectrum was simulated by using Bruker's WinDAISY program [Ernst, 2009].

4. Results and discussion

In this chapter, the results of the experiments are displayed, explained and compared with other published data, if found. These experiments were focused mainly on sorption and desorption behavior of diclofenac at different concentration levels, biotransformation and phototransformation fate of diclofenac in water/sediment systems with different experimental design, identification of phototransformation products of diclofenac and isolation of the most abundant one.

4.1 Sorption/desorption behavior of diclofenac in sandy loam sediment

In order to describe the affinity of diclofenac to sediments, sorption and desorption experiments were performed. By means of these experiments, mobility, leaching tendency and remobilization of diclofenac can be estimated. Based on the concentration decrease in the aqueous phase (indirect method), sorption coefficients (K_d) of diclofenac onto sandy loam sediment (S3) were calculated. The results showed K_d of 5.5 ± 0.8 **[Table 8]**. The complete data set are listed in **Appendix, Table A9**. The accuracy of the results was tested by multiplying the K_d values with the sediment/solution ratio which were found to be > 0.3 L/kg. Therefore, the estimated K_d values based on the current indirect method were considered to be accurate and there was no need to analyze the sediment phase. The latter is recommended to be analyzed as well, when the K_d value or the result of its multiplication with the sediment/solution ratio is < 0.3 L/kg [OECD, 2002b].

The estimated K_d value revealed that diclofenac could be considered as a slightly mobile pharmaceutical compound. Only pharmaceuticals with $K_d \leqq 1$ L/kg are considered to be quantitatively mobile. Therefore, leaching tendency of diclofenac could be expected to some extend through the sediment under investigation. Since concrete leaching potential standards of pharmaceuticals in sediments have not been set yet, alternatively, the leaching potential criteria of pesticides through the soil can be temporarily used for predicting the transport of diclofenac. Enhanced leaching potential of pesticides in soil were defined by K_d < 5, K_{oc} < 300, water solubility >30 mg/L, half life in soil >14 days, and half life in water >175 days [Fichter and Holden, 1992].

Additionally, it is well known that the texture of the sorbent material extremely affects the sorption process. In batch experiments using sandy sediment, the K_d values of diclofenac reported by Scheytt et al. (2005a) were in the range of 0.6 to 4.7 L/kg. In silty sand and clayey silt soil, Kreuzig et al. (2003) reported K_d values of 4 and 10 L/kg, respectively. In soil leaching experiment, Drillia et al. (2003) observed no recovery of diclofenac in the leachate

due to the strong sorption of diclofenac onto the soil. However, depending on other conducted laboratory soil column experiments [Mersmann et al., 2002, Scheytt et al., 2004] higher mobility of diclofenac under natural aquifer conditions than calculated from octanol/water partition coefficients was expected. In any case, the present experiments indicate that even for the sediments with low organic carbon sorption of negatively charged substances (polar) is considered a relevant process.

Table 8: Distribution coefficient K_d and Freundlich parameters

	K_d (L/kg)	K_F ($\mu g^{1-1/n}$ $L^{1/n}$/kg)	1/n	R^2
Sorption	5.5 ± 0.8	7.5 ± 1.0	0.92	0.99
Desorption	13.3 ± 3.0	15.8 ± 1.0	0.90	0.90

In order to study the sorption behavior of diclofenac in more depth, sorption isotherm was constructed at constant temperature. There are mainly two common models describing the sorption processes i.e., Freundlich isotherms which originally derived for describing the adsorption of gas molecules on the solid surface and Langmuir isotherms which originally derived for describing the adsorption of gas molecules forming monolayer on the surface of solid. These models are traditionally and successfully used for describing the adsorption of pharmaceutical from aqueous solutions onto solids as well [Ternes et al., 2002, Scheytt et al., 2005a, Hajare and Pishawikar, 2006, Tella and Owalude, 2007, Ramil et al., 2010].

The sorption data of diclofenac showed that the isotherms were concentration dependent and best fitted to the Freundlich isotherm model. The relationship of $\log C_s$ against $\log C_w$ is shown in **Figure 14**. From which, the Freundlich affinity constant (K_F) and the exponent (1/n) were calculated from the intercept and slope of the generated isotherm, respectively. The entire Freundlich parameters are represented in **Table 8**. The Freundlich exponent 1/n was found to be close to 1.0 indicating that the sorption isotherm of ^{14}C-diclofenac are almost linear which means increasing sorption affinities with increasing aqueous phase concentrations of ^{14}C-diclofenac. The K_F value of 7.5 ± 1 was slightly higher compared to the K_d values. This variation is mostly due to the value of 1/n (0.92) which is not exactly equal the unity. The Freundlich isotherm becomes linear when 1/n = 1. This is the simplest case of the Freundlich isotherm and is named the Nernst partitioning (K_d). In this case, the Freundlich adsorption coefficient (K_F) will be equal to the distribution coefficient (K_d).

The K_F value presented in this study differs from previous sorption experiments made by Ternes et al. (2002) and Scheytt et al. (2005a). For elimination of diclofenac from drinking water, Ternes et al. (2002) used granular activated carbon as a solid phase showing higher K_F values ranging from 36 to 141. Compared to K_F presented in the current study, lower values (0.57-0.81) were calculated by Scheytt et al. (2005a). Again the reason may be the difference in the texture of the sediments where sandy sediment was used during those experiments while sandy loam sediment was used in the present study. Sorption properties of the sediments are influenced mainly by TOC, clay, and silt content. However, substances not only partition into the organic matter but also a certain fraction of their molecules "sorbs" to the internal microvoids or holes [Stein et al., 2007]. Furthermore, sorption can be attributed to several different mechanisms, namely adsorption or absorption to the natural organic matter, interactions with mineral surfaces, bonding to several different kinds of surface moieties, and interactions of charged species with solids [Scheytt et al., 2005a, Schwarzenbach et al., 2003].

Since desorption processes also play an important role in the behavior of chemicals in the environment, the desorption coefficient (K_{des}) was calculated **[Table 8]** and the Freundlich desorption isotherm was constructed **[Figure 15]**. The results showed that the K_{des} was 13.3 ± 3 which was found to be much higher than the sorption coefficient (K_d). The complete data set are listed in **Appendix, Table A10.** The results reflected higher apparent affinity of diclofenac to the sediment in the desorption direction than in the sorption direction. Additionally, desorption percentage was found to be 20 ± 3 % indicating that ^{14}C-diclofenac was irreversibly sorbed onto the sediment. Low desorption values could further limit diclofenac migration or mobilization in surface waters. Desorption is considered reversible when the amount desorbed is more than 75 % of the amount sorbed [OECD, 2002b]. The Freundlich desorption isotherm of diclofenac was slightly linear (1/n = 0.9) and the $K_{F\text{-}des}$ was 15.8 ± 1.

For the purposes of comparison, additionally, surface water was used as a solvent phase instead of $CaCl_2$ solution. The K_d and K_{des} of surface water were found to be 4.4 ± 0.6 and 15.8 ± 1.7, respectively. These results reveal no considerable difference between $CaCl_2$ and surface water as aqueous solvent phases. On the other hand, K_{oc} (organic carbon normalized adsorption coefficient) was not calculated in the present study because it is only to some extent applicable to polar substances and not applicable with sediment of low organic matters content [Fränzle et al., 1987, Calvet, 1989, OECD 2002b].

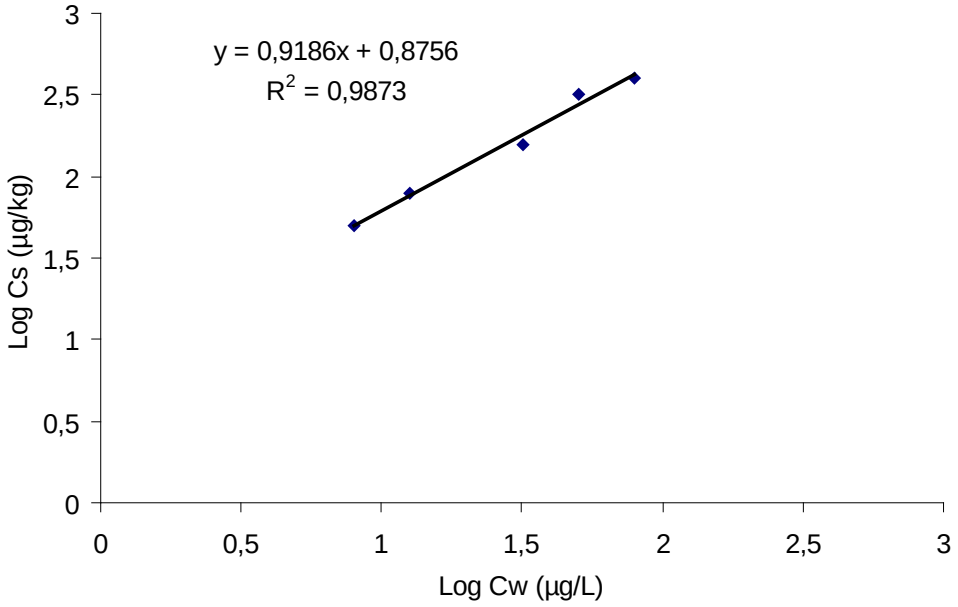


Figure 14: Freundlich sorption isotherms of diclofenac onto sandy loam sediment (S3). C_s and C_w are the sorbed and aqueous concentrations, respectively

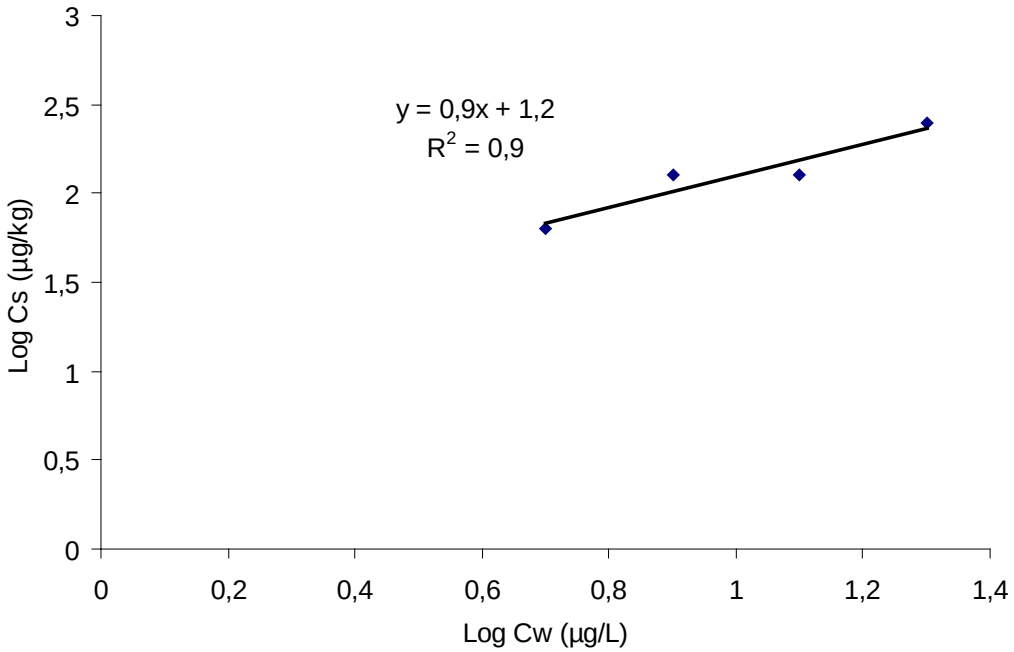


Figure 15: Freundlich desorption isotherms of diclofenac from sandy loam sediment (S3). C_s and C_w are the remaining adsorbed and desorbed aqueous concentrations, respectively

4.2 Fate monitoring of radiotracer diclofenac in dark water/sediment systems

In the dark, the microbial activity is considered the major cause of the disappearance of diclofenac from the water/sediment systems. The reason could be the biotransformation processes which expected to occur at the interface between water and sediment [Craven et al., 1986]. However, sorption onto the sediment and diffusion controlled transport could be other reasons. Therefore, for a better understanding, the disappearance of diclofenac can be differentiated into mineralization, formation of transformation products either in the aqueous phase or in the extractable residues (ER) and formation of non-extractable (NER) or bound residues. The boundary conditions, in which the degradation processes occurred, were obtained through characterization of the used water and sediments matrices at the termination of each incubation interval.

4.2.1 Matrix characterization

In both sediments (S1 and S2) as well as in both laboratory test systems, i.e., batch system and the biometric flask equipped with soda lime trap system, the physico-chemical parameters such as pH and the redox potential of water and sediments, the oxygen concentration in the water phase and TOC in the sediments generally displayed no considerable variations at the beginning and at the end of the study **[Table 9, 10]**. Directly after sampling, both water and sediments were found to be slightly alkaline. The pH values of the water, S1 and S2 were 8.3, 7.4, and 7.3, respectively. These values remained nearly constant until the end of the dark experiment. During the study period, aerobic conditions were indicated by the Eh and O_2 content values which were almost higher than 150 mV and 2 mg/L, respectively. However, Eh < 150 mV were observed at the end of some incubation intervals. This observation could be caused by interferences of the electrode measurement in suspended matter [Kreuzig et al., 2007]. Particularly for the biometric flasks with soda lime, applied under continuous gas exchange, Eh < 150 mV is not realistic. Additionally, the TOC values of both sediments were found to be relatively low. The sediments were found to be microbially active as indicated by their SIR values that were between 1.0-1.9 mg O_2/100 g h and 1.2-3.4 mg O_2/100 g h for S1 and S2, respectively, during the course of the experiments. As shown from the control samples, the organic solvent used in the fortification of the test substance had no effect on the microbial activity of both sediments within the study period.

Table 9: Physico-chemical parameters of water/sediment WS1 systems used in transformation test of diclofenac in the dark

	Water				Sediment			
	pH	Eh [mV]	O_2 [mg/L]	TOC [mg/L]	pH	Eh [mV]	SIR [mg O_2/100 g h]	TOC [% ds]
AS	8.3	430	7.8	49.3	7.4	210	1.9	0.2
BE	8.2	410	6.1	70.1	7.6	310	1.0	0.2
d0 BS	8.3	410	4.7	---	7.5	380	---	---
d1 BS	8.0	430	2.8	---	7.6	230	---	---
d3 BS	7.9	420	2.4	---	7.6	10	---	---
d7 BS	7.6	380	2.4	---	7.5	30	---	---
d14 BS	7.7	380	3.2	---	7.5	-30	---	---
d28 BS	7.6	430	3.9	---	7.3	90	---	---
d56 BS	7.8	430	4.4	---	7.1	170	---	---
d100 BS	7.7	420	5.1	155.5	7.2	100	1.5	0.3
d100 BS Control	7.7	430	5.1	107.7	7.1	150	1.2	0.2
d3 SLT	8.0	390	1.9	---	7.6	-20	---	---
d56 SLT	8.3	400	4.4	---	7.3	110	---	---
d100 SLT	8.5	440	5.1	172.2	7.8	130	1.6	0.3
d100 SLT Control	8.2	470	5	114.3	7.5	200	1.3	0.2

AS = after sampling, BE = before equilibration, BS = batch systems, SLT = flasks equipped with soda lime trap systems, d = day(s), ds = dry substance, --- = not determined

Table 10: Physico-chemical parameters of water/sediment WS2 systems used in transformation test of diclofenac in the dark

	Water				Sediment			
	pH	Eh [mV]	O_2 [mg/L]	TOC [mg/L]	pH	Eh [mV]	SIR [mg O_2/100 g h]	TOC [%.ds]
AS	8.3	430	7.4	67.4	7.3	220	1.9	0.4
BE	7.8	370	6.0	90.5	7.1	270	1.2	0.4
d0 BS	7.5	440	2.1	---	7.1	130	---	---
d1 BS	7.7	420	2.2	---	7.4	330	---	---
d3 BS	7.4	400	2	---	7.1	50	---	---
d7 BS	7.5	390	1.7	---	7.2	80	---	---
d14 BS	7.7	420	3	---	7.1	10	---	---
d28 BS	7.6	420	4.3	---	7.3	20	---	---
d56 BS	7.8	440	4.7	---	7.4	170	---	---
d100 BS	7.8	440	5.2	240.8	7.5	200	2.1	0.5
d100 BS Control	7.8	440	5.2	133.2	7.3	210	3.3	0.4
d3 SLT	7.4	430	2.3	---	7.2	60	---	---
d56 SLT	8.3	430	5.1	---	7.6	200	---	---
d100 SLT	8.5	410	5.2	230.1	7.8	210	3.4	0.5
d100 SLT Control	8.5	410	5.1	140.3	7.9	200	3.4	0.4

AS = after sampling, BE = before equilibration, BS = batch systems, SLT = biometric flasks equipped with soda lime trap systems, d= day(s), ds = dry substance, --- = not determined

4.2.2 Mineralization rates

The most efficient elimination process for every organic compound in the environment is complete mineralization (MIN) by microorganisms to carbon dioxide and water. In the present study, diclofenac showed mineralization rates of 13 ± 0.2 % and 13 ± 0.1 % of the initially applied radioactivity amount in case of WS1 and WS2, respectively, after 100 d incubation period in the batch system. Within the incubation period in the biometric flask system, diclofenac was mineralized to 13 ± 3.5 and 13 ± 1.8 % of the initially applied radioactivity amount in case of WS1 and WS2, respectively. These results indicated that mineralization of diclofenac to carbon dioxide and water occurred at lower amounts.

It has to be noticed that the biometric flask system had an obvious disadvantage where the analysis of the radioactivity amounts trapped by the soda lime granules was laborious. In contrast, the analysis of potassium hydroxide trap solution of the batch system was much easier.

4.2.3 Disappearance of ^{14}C-diclofenac from the aqueous phases

The investigation of aqueous phase (AP) of water/sediment systems is very important for estimation of the behavior of diclofenac. In the batch system, it was observed that the radioactivity disappeared out of the aqueous phase during the study period. For the WS1, the radioactivity decreased from 106 ± 0.5 % to 10 ± 0.6 % of the initially applied radioactivity amount within an incubation period of 100 days **[Figure 16]**. By the same way, the radioactivity in the water phase of WS2 decreased from 106 ± 1.5 % to 9 ± 0.2 % of the initially applied radioactivity amount within an incubation period of 100 days **[Figure 17]**.

As shown by RTLC, the biotransformation of diclofenac was started, probably at the water/sediment interface, without any lag period and almost completely disappeared after 28 d and 56 d in WS1 and WS2 **[Figure 18]**, where 1.3 % and 5.2 % could be detected as diclofenac, respectively. During these periods, the first solvent (n-hexane/ethyl acetate/acetic acid; 50:50:1 v/v/v) and the second solvent (dichloromethane/methanol/25 % ammonia; 85:14:1 v/v/v) showed formation of several unknown biotransformation products. An example is given in **Figure 19**. The numbers of the unknowns differed according to the incubation interval of each sediment type **[Appendix, Table A11-A14]**. On the other hand, mainly one of these biotransformation products could be detected at the termination of the experiments suggesting further transformation for those products. The elimination rate constants of diclofenac from the water phase were calculated to be 0.07 and 0.05 per day resulting in DT_{50} of 10 and 13 d and DT_{90} of 34 and 42 d for WS1 and WS2, respectively.

It has to be noted that, the radioactivity amount regarding diclofenac in the water phase of WS1 (44 %) and WS2 (36 %) exhibited no change in the test samples of 7 d and 14 d as shown by RTLC. This observation could be an indication on the ecotoxicity of the biotransformation products of diclofenac due to the susceptibility of the biomass against these biotransformation products. As a result, the microorganisms utilizing diclofenac as the energy source could be inhibited. On this basis, stopping of the biotransformation process might be expected and after a recovery period the biotransformation process could be continued. Additionally, the radioactivity amounts detected in all compartments, in the WS1, within the incubation period of 7 d and 14 d, were found to be almost the same. The previous observation may refer to the close relationship between the biotransformation process and the formation of the non-extractable residues. The same observation has been reported by Kreuzig et al. (2003). They confirmed that the formation of non-extractable residues during the investigation of diclofenac in soil is directly depending on the microbial activity.

Despite the microbial activity at the end of the experiment was found to be relatively higher in the WS2 sediment (2.1 and 3.4 mg O_2/100 g h for batch and biometric flask systems, respectively) than in the WS1 (1.5 and 1.6 mg O_2/100 g h for batch and biometric flask systems, respectively), the disappearance of diclofenac from the water phase of the WS1 was found to be faster than in the water phase of WS2 **[Figure 18]**. It was found that, after 28 d 1 and 21 % of the initially applied radioactivity could be identified as diclofenac in the WS1 and WS2, respectively. These results showed that there was no relation between the total microbial activity and the biotransformation rate of diclofenac, i.e., the degradation of diclofenac was found to independent on the total microbial population. This observation might indicate that biotransformation of diclofenac is restricted to specific group(s) of microorganisms, an assumption which is supported by observations of Paje et al. (2002) and Groening et al. (2007), or it is restricted to a specific enzyme produced by certain microorganisms during the metabolism of another compound which might support the idea of the co-metabolic nature of diclofenac [Groening et al., 2007]. The latter observed that presence of Gram-negative bacteria, especially those belonging to the Cytophaga-Flavobacterium group and the γ-Proteobacteria, cause an increase of diclofenac biotransformation.

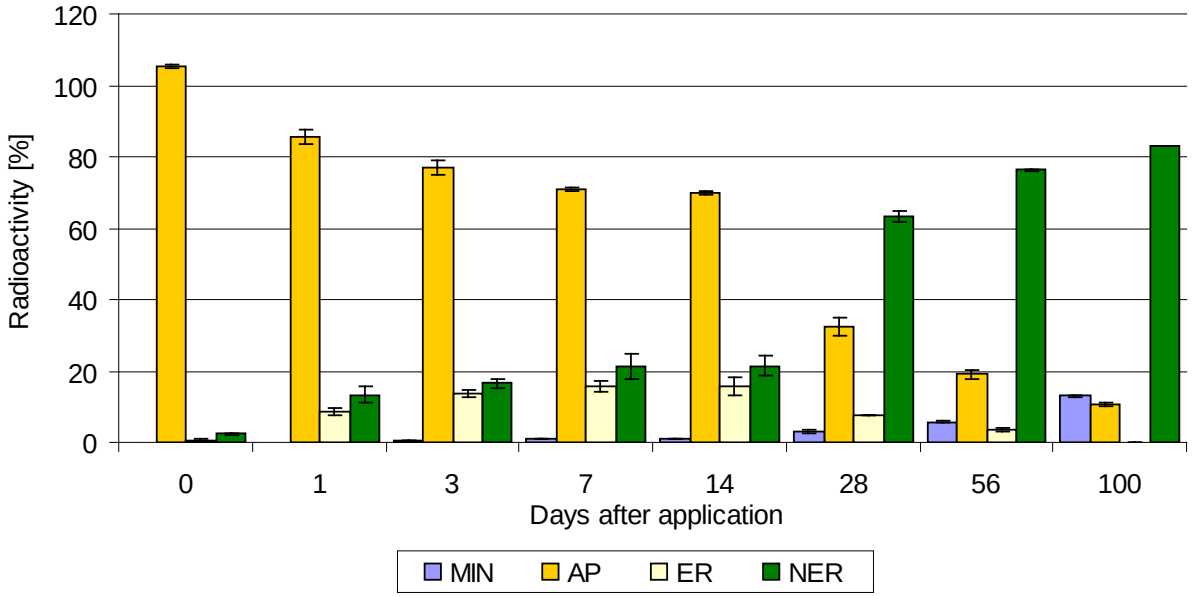


Figure 16: Balances of the biotransformation test series of ^{14}C-diclofenac in water/sediment WS1 system using laboratory batch systems

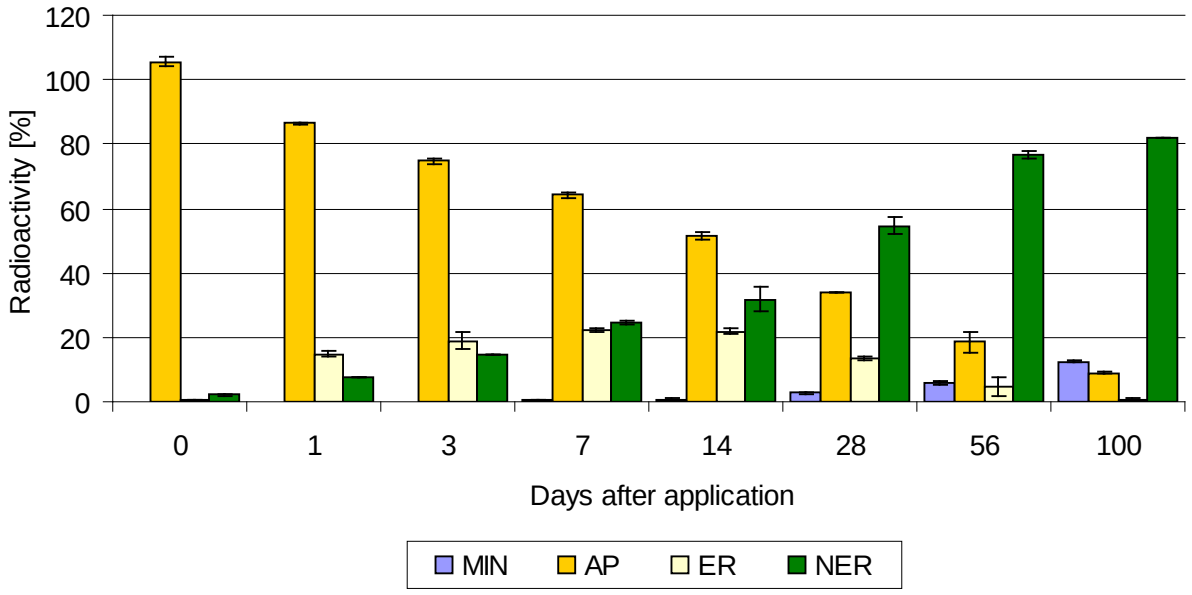


Figure 17: Balances of the biotransformation test series of ^{14}C-diclofenac in water/sediment WS2 system using laboratory batch systems

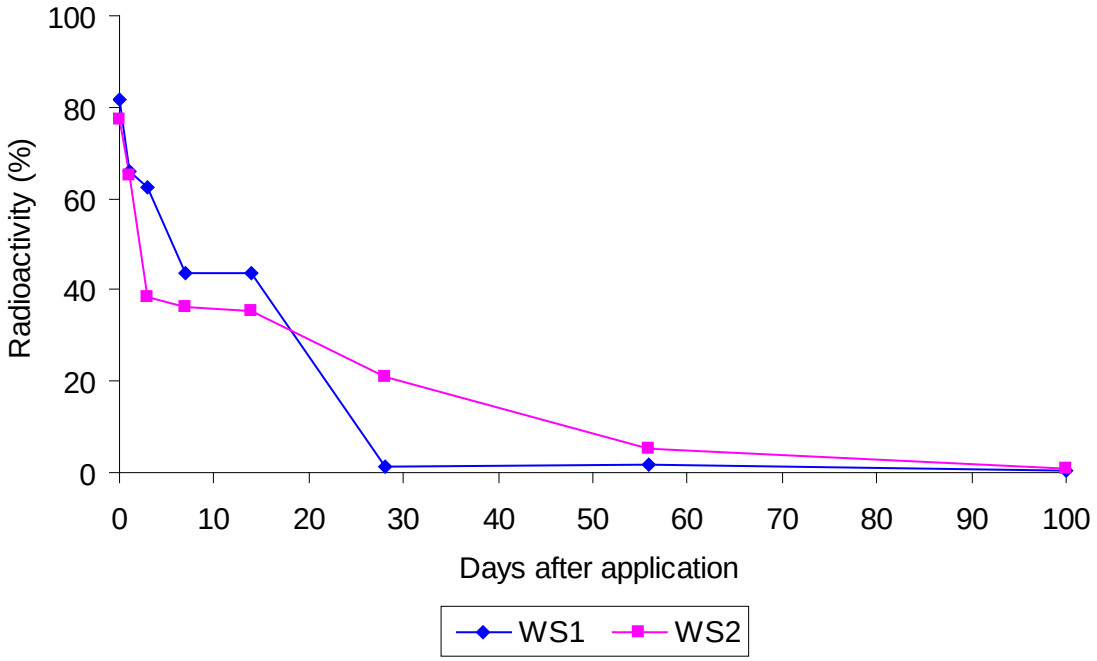


Figure 18: **Disappearance of ^{14}C-diclofenac from the water phases of the laboratory batch system water/sediment WS1 and water/sediment WS2 within 100-d incubation period in the dark as shown by the first solvent (n-hexane/ethyl acetate/acetic acid, 50:50:1 v/v/v)**

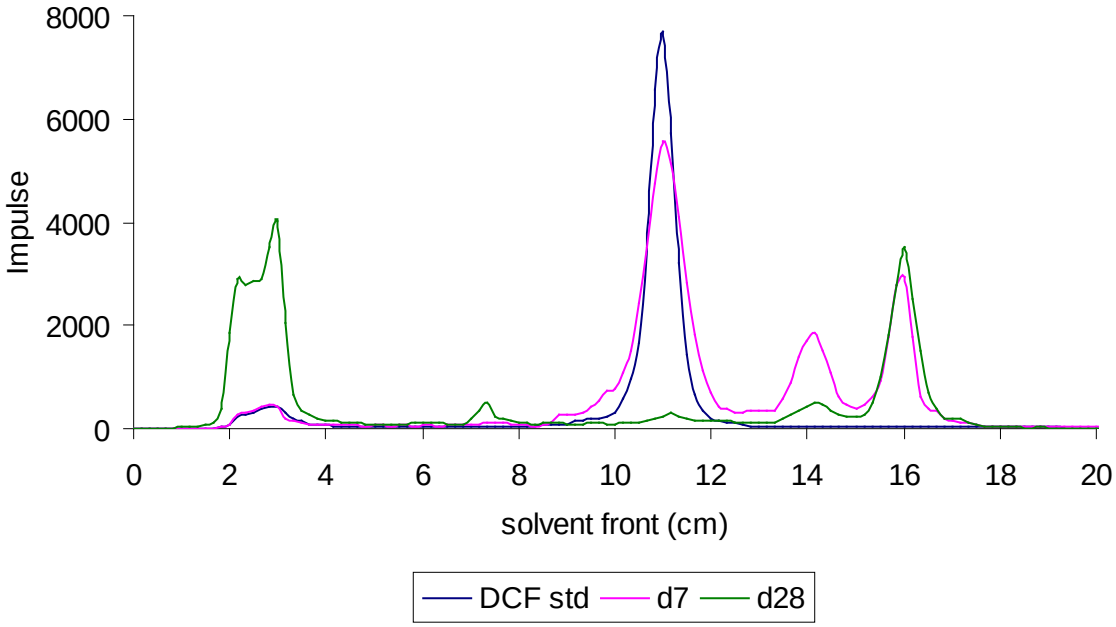


Figure 19: **RTLC chromatogram of ^{14}C-diclofenac (DCF) in the water phase of the laboratory batch system WS1 incubated in the dark for different intervals, extracted by SPE and developed by first solvent (n-hexane/ethyl acetate/acetic acid, 50:50:1 v/v/v)**

4.2.4 Extractable residues

The extractable residues (ER) represent the fractions of diclofenac and/or its biotransformation products that could be extracted by means of an organic solvent. According to their polarity and eluotropic series against silica gel, methanol, acetonitrile and acetone are classified as polar organic solvents with elution strength of 0.73, 0.50, and 0.47, respectively. They are miscible with water in all ratios. In contrast, the miscibility of ethyl acetate (elution strength = 0.38), as a less polar organic solvent, with water is only 8.7 % (w/w). Basically, water miscible organic solvents are expected to exhibit higher extraction capacity for diclofenac from wet sediments than solvents partially miscible with water. However, the test of extraction efficiency showed that ethyl acetate had the highest extraction power (90 ± 3.5) in comparison to acetone (69 ± 4.7), acetonitrile (69 ± 3.6) and methanol (57 ± 2.9) as shown in **Figure 20**. The complete data set are listed in **Appendix, Table A15**. Additionally, ethyl acetate provides minimal disturbance for the water/sediment interface which considered the most important layer for biotransformation processes [Craven et al., 1986]. Among the tested solvents, moreover, ethyl acetate was found to be the easiest organic solvent in handling. Therefore, ethyl acetate was chosen as the most suitable solvent for the extraction process.

Sorption or weakly interaction of diclofenac and its biotransformation products with the sediments, probably by van der Waals forces, could be the cause of the extractable fraction. Additionally, pore water of wet sediment could be also involved as a reason of formation of the extractable residues. As shown in **Figure 21**, two different extraction methods for water/sediment samples were compared for calculation of the radioactivity amounts that could be remained in the pore water. After separation of the water/sediment system, therefore, sediments were either extracted directly by ethyl acetate or rinsed with fresh water three times (50 mL each) before ethyl acetate extraction step. The difference between the radioactivity amounts detected in the ER of both methods (10 %) was equivalent to the effect of pore water **[Appendix, Table A16]**. It is obvious that during the rinsing process the sediment surface is completely disturbed. Such disturbance is not recommended either by guideline 308 [OECD, 2002a], or by Löffler et al. (2005) or Ericson, (2007). Therefore, the radioactivity amounts of the pore waters were added to the extractable fractions within the present study.

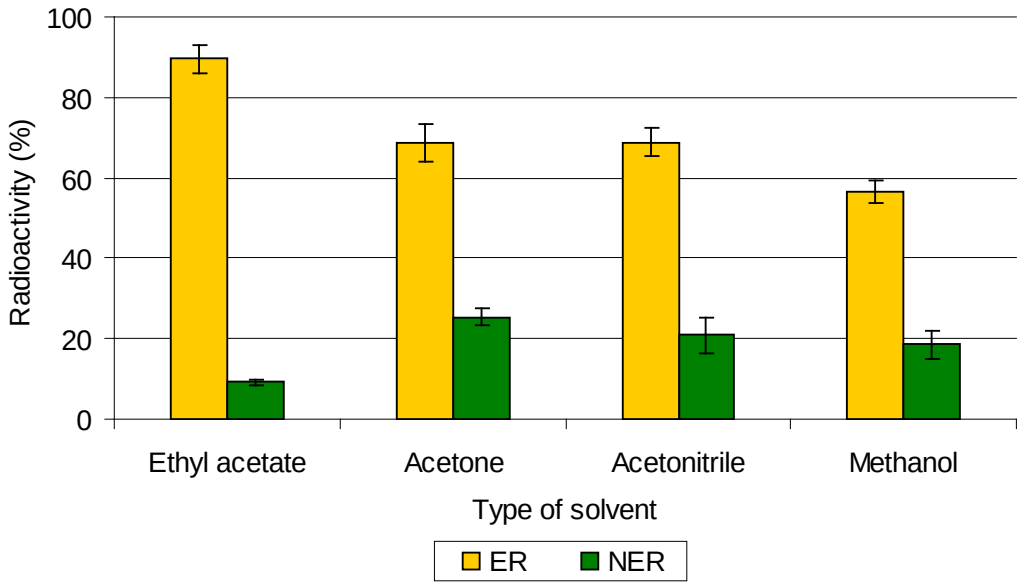


Figure 20: Extraction efficiency tests for ^{14}C-diclofenac in wet sediment (S3) using different solvents

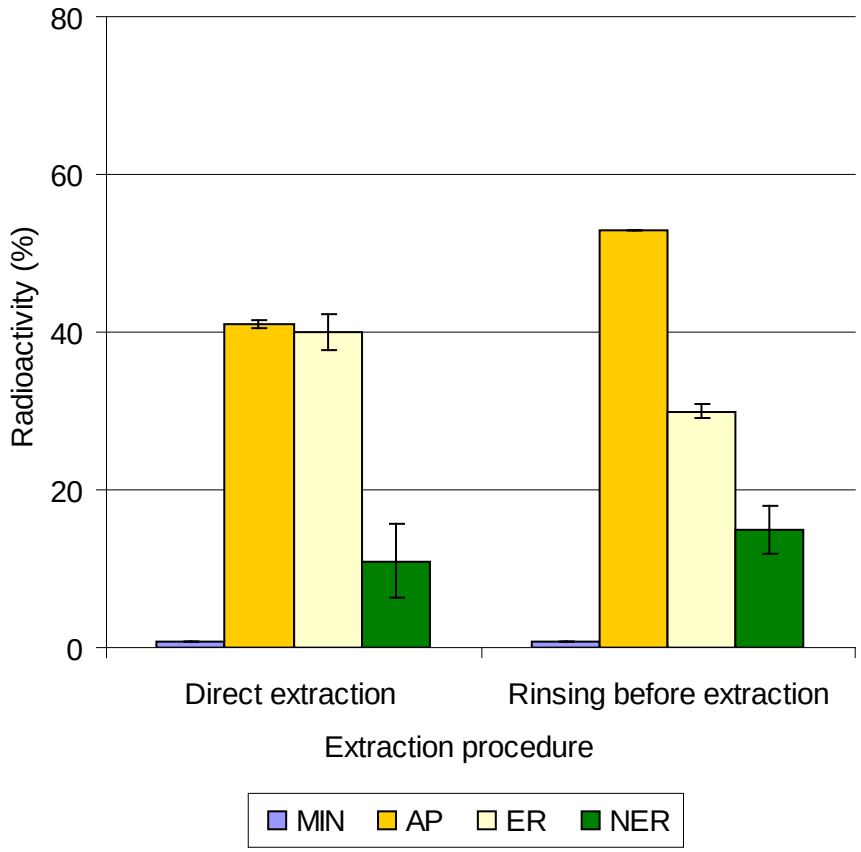


Figure 21: Tests on remaining radioactivity of ^{14}C-diclofenac in pore water after direct solvent extraction

According to the time of contact, abundance of the biotransformation products and the affinity of diclofenac and its biotransformation products for sorption onto the sediments, the radioactivity amounts that could be extracted with ethyl acetate were determined. Ethyl acetate extracts were found to contain traces of the initially applied radioactivity at the beginning (0.7 ± 0.1 and 0.8 ± 0.1 % for WS1 and WS2 sediments, respectively), and at the termination of the experiments (0.2 ± 0.1 and 0.8 ± 0.1 % for WS1 and WS2 sediments, respectively) **[Figure 16, 17]**. These results may indicate that, at the beginning of incubation intervals, the time of contact either between diclofenac which has already moderate sorption properties or its biotransformation products which may have higher sorption properties than diclofenac and the sediments which have already low organic matter content was not enough for strong interaction between them. Therefore, the sediments were not able to keep high amounts of radiotracer substances and hence low radioactivity amounts were detected in the extractable residues. On the other hand, at the termination of the incubation intervals, diclofenac was completely transformed biologically to other products as shown by RTLC. These products had enough time to interact strongly, probably by sorption, with the sediments. As a result of this strong sorption process, ethyl acetate was not able to extract these biotransformation products from the sediments. Therefore, low radioactivity amounts were detected in the extractable residues. Between the beginning and the end of the incubation intervals, diclofenac might be weakly sorbed onto the sediment and hence the extractable residues increased as the time of contact increased up to 14 d. After this time, the extractable resides continuously decreased as the amounts of diclofenac decreased due to the biotransformation process.

This finding clarified that only biotransformation products of diclofenac had the capability to interact strongly with the sediments. This assumption was supported by the results of the 7-d and 14-d incubation intervals in which the extractable residues contained almost 16 and 22 % from the initially applied radioactivity for WS1 and WS2, respectively. Most of these amounts, 10 and 14 % as shown by the first solvent and the second solvent, respectively, in case of WS1 and 15 and 20 % as shown by the first solvent and the second solvent, respectively, in case of WS2, were identified by means of RTLC as the parent compound **[Appendix, Table A17-A20]**. Finding of mainly diclofenac in the extractable residues might be attributed to a moderate sorption process onto the sediments.

Sorption of diclofenac to the sediments can not be explained mainly by its lipophilicity (P_{ow} 4.51) because under the pH conditions in sediment (pH 7.4) and water (pH 8.3) diclofenac (pKa 4.16) was relatively present in its negatively dissociated form due to the presence of an

ionizable carboxyl group. Therefore, the occurrence of diclofenac in the sediment can be expected to be caused mainly by ionic interactions [Scheytt et al., 2005a]. The ionic sorption could occur on positively charged sediments minerals. Hydroxy-iron and -aluminum compounds exhibit a high sorption capacity for acids. This could be attributed to the large chemical reactivity of these positively charged minerals toward the negatively charged carboxyl and hydroxyl groups of the acid compounds. Additionally, sediment organic functional groups have considerable role in the interaction processes [Spadotto and Hornsby, 2003, Huang et al., 1977]. On the other hand, Chefetz et al. (2008) proved that the organic matter have a considerable effect in the sorption process of diclofenac onto soil. The presence of diphenyl moiety in the structure of diclofenac may play an important role in the interaction of diclofenac with the solid organic matter.

In the present study, sorption unlikely be attributed to the organic matter where the TOC content was found to be relatively low in both sediments (0.2 and 0.4 % in the S1 and S2 sediments, respectively) **[Table 9, 10]**. The clay content of both sediments could play a role in the sorption process where the extractable residues were found to be higher in the WS2 than in the WS1 **[Figure 16, 17]**. The reason could be due to the higher percentage of the clay content in case of S2 sediment (30 %) than in case of the S1 sediment (8.5 %) **[Table 5]**. However, because of the major amount of diclofenac has been detected in the water phase, the microbial transformation expected to represent the most dominant sink for diclofenac in the dark.

In contrast to other incubation intervals, the RTLC of the concentrated ethyl acetate extracts of 56 d and 100 d incubation intervals in both sediments were found to be difficult for integration, using both solvents, due to the low radioactivity amounts, at maximum 5 % of the initially applied radioactivity for both sediments.

4.2.5 Non-extractable residues

The amounts of the radioactivity remained after the ethyl acetate extractions in both sediments were considered as non-extractable residue (NER). It was found, that the non-extractable residues increased from 2 ± 0.1 to 83 % and from 2 ± 0.4 to 82 ± 0.1 % for S1 and S2, respectively, within an incubation period of 100 days **[Figure 16, 17]**. Strong sorption or binding to the sediments by different types of mechanisms such as hydrogen bonds, charge transfer complexes and covalent bonds could be the reason for formation of the NER [Haider et al., 1992, Northcott and Jones, 2000, Gevao et al., 2000]. Additionally, trapping of molecules in a molecular sieve formed by humic materials could be also another possible reason [Haider et al., 1992]. These mechanisms are usually results in strong irreversible interaction that could be attributed to the formation of highly reactive intermediate biotransformation products which interacts strongly with the sediments.

Additionally, formation of volatile biotransformation products was relatively excluded as the radioactivity recovered was entirely complete at all incubation intervals with a median mass balance of 106 ± 2. The complete data sets are represented in **Appendix, Table A21, A22**.

Briefly, the fate of diclofenac in water/sediment systems almost resulted in mineralization rate of 13 %, DT_{50} in the water phase of 13 d, and formation of 82 % non-extractable residues within of 100-d incubation period. By means of such data, the persistence criteria of diclofenac in water/sediment systems can be estimated in different ways. According to the European Union for the pesticide registration procedure, persistence can be achieved by mineralization < 5 %, DT_{50} > 120 d, and formation of non-extractable residues > 70 % [Federal Biological Research Center for Agriculture and Forestry, 1998, European Commission, 2006]. On this basis, diclofenac can be considered as a moderately persistent organic compound. According to the environmental classification of OECD guideline 308 [OECD 2002a], on the other hand, diclofenac can be classified as a non-persistence pharmaceutical compound where pharmaceuticals that have DT_{50} < 16 d, 16 < DT_{50} < 40 d, and DT_{50} > 40 d are classified as non-persistent, moderately persistent, and potentially persistent compounds, respectively.

4.2.6 Comparison between the batch system and the biometric flask system

In order to evaluate the applicability of the batch system, the discontinuous gas exchange system, a comparison with the biometric flask system, the continuous gas exchange system, was performed for both sediment types. Three incubation intervals, i.e., 3 d, 56 d, and 100 d of the experiments, were selected to be compared with each other. This comparison was estimated depending on the redox potential, oxygen content, radioactivity amounts detected in each compartment, the dissipation behavior of diclofenac as detected by RTLC, and the mass balances obtained at the termination of the incubation intervals of both water/sediment systems. In all water/sediment samples, the redox potential in the water phase was found to be ≥ 390 mV. At the termination of the experiments, additionally, the oxygen concentrations were ≥ 5 mg/L. Theses results indicated that, the biotransformation processes of diclofenac in both systems were performed under aerobic conditions. Especially for 56 d and 100 d incubation intervals, furthermore, the detected total radioactivity amounts in all compartments, the mass balances **[Figure 22, 23]** and the radioactivity amount detected as diclofenac in the water phase **[Table 11]**, especially for WS1, were found to be close to each other. The complete data set are listed in **Appendix, Table A23, A24**.

Table 11: Percentage of radioactivity remained as diclofenac in the water phase of the applied water/sediment systems

	Water phase							
	WS1				**WS2**			
	1st solvent		**2nd solvent**		**1st solvent**		**2nd solvent**	
	BS	**SLT**	**BS**	**SLT**	**BS**	**SLT**	**BS**	**SLT**
3 d	63.0	61.0	66.0	69.0	38.0	55.0	38.0	67.8
56 d	2.0	2.0	nd	nd	5.2	5.0	9.0	10.0
100 d	0.5	0.9	nd	nd	0.7	2.0	2.0	2.0

1st solvent = n-hexane/ethyl acetate/acetic acid (50:50:1 v/v/v), 2nd solvent = dichloromethane/methanol/25 % ammonia (85:14:1 v/v/v), nd = not determined

Moreover, the RTLC of the extractable residues showed almost identical results within 3 d incubation interval. For WS1, diclofenac was disappeared to 9 %, when developed by the first solvent, and up to 12 %, when developed by the second solvent, for the batch and the biometric flask systems, respectively. For WS2, disappearance up to 14 % was detected for

diclofenac by the first solvent and up to 16 % by the second solvent for the batch and the biometric flask systems, respectively. In all cases, on the other hand, the RTLC of the concentrated ethyl acetate extracts of 56 d and 100 d incubation intervals were found to be difficult for integration due to the low radioactivity amounts, i.e., 5 % of the initially applied radioactivity for both sediments. This comparison clearly showed that both laboratory test systems lead to equivalent results on fate of ^{14}C-diclofenac in water/sediment systems.

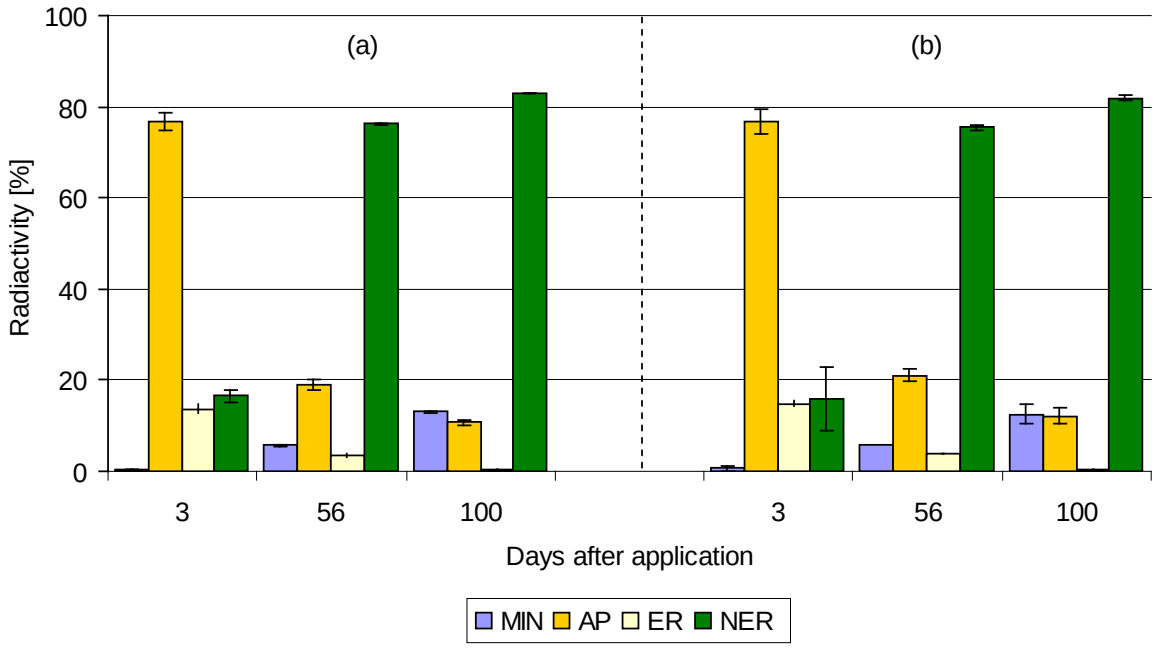


Figure 22: Balances of the biotransformation tests of ^{14}C-diclofenac in water/ sediment WS1 after incubation in the dark for different intervals using (a) laboratory batch system and (b) biometric flask system

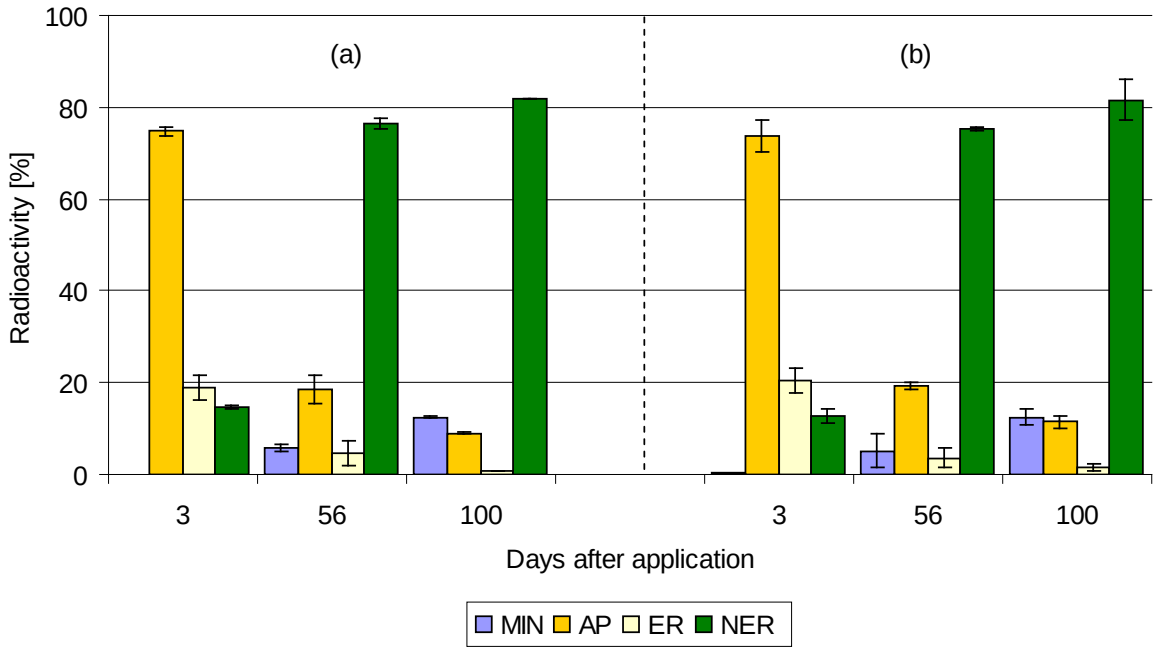


Figure 23: Balances of the biotransformation tests of ^{14}C-diclofenac in water/ sediment WS2 after incubation in the dark for different intervals using (a) laboratory batch system and (b) biometric flask system

4.3. Fate monitoring of ^{14}C-diclofenac after irradiation in water/sediment systems

Under the influence of light the behavior of diclofenac, as a photosensitive compound, in water/sediment system was completely unknown. Therefore, irradiation experiments were conducted using two different types of sediments in order to simulate the effect of natural light on diclofenac in water/sediment systems. After 10 h/d irradiation of diclofenac for 3 consecutive days, the radioactivity amounts detected as non-extractable residues in both sediments showed unexpected high amounts. From the initially applied radioactivity, 65 ± 1.8 and 70 ± 0.1 % were localized as non-extractable radioactivity in the sediments of WS1 and WS2, respectively **[Figure 24]**. This could be due to the formation of high reactive intermediate products during the irradiation time [Agueera et al., 2005] as a result of removal of the chlorine atom from the phenyl ring of diclofenac. These intermediate products might interact strongly with the sediment leading to formation of highly stable complexes that could not be extracted by means of ethyl acetate. Mineralization was only observed to minor extent of 1 ± 0.2 % for both WS systems. The radioactivity amounts in the water phases were 35 ± 4 and 26 ± 4 % for WS1 and WS2, respectively, from which about 2 % for both WS systems could be identified as diclofenac and the remaining radioactivities were mainly detected at the spot of application as a very large peak (29 and 16 % for WS1 and WS2, respectively) **[Figure 25]**. It could be a mixture of different highly polar primary or secondary phototransformation products.

The previous results are consistent with the photosensitivity phenomena of diclofenac considering the photolysis as the main fate of diclofenac in presence of light. Furthermore, the mass balance showed that the radioactivity was recovered in an acceptable level (104 ± 3 and 107 ± 5 for WS1 and WS2 sediments, respectively). In contrast, irradiation of diclofenac in pure water/quartz sand (QS) sediment system showed formation of 19 % as non-extractable residues while the major amount (66 %) of the initially applied radioactivity was detected in water phase. The mass balance was found to be 96 % from which 7 % could be detected in the water phase as ^{14}C-diclofenac, as shown by RTLC. The complete data set are listed in **Appendix, Table A25, A26**. In all cases, the extractable residues were found to be difficult for integration by RTLC due to the low radioactivity amounts (10 % at maximum). These results revealed that the difference between the water/quartz sand system and the other water/sediment systems affected the distribution behavior of diclofenac in their compartments. On the other hand, the microbial activity had negligible effect on the transformation of diclofenac in presence of light

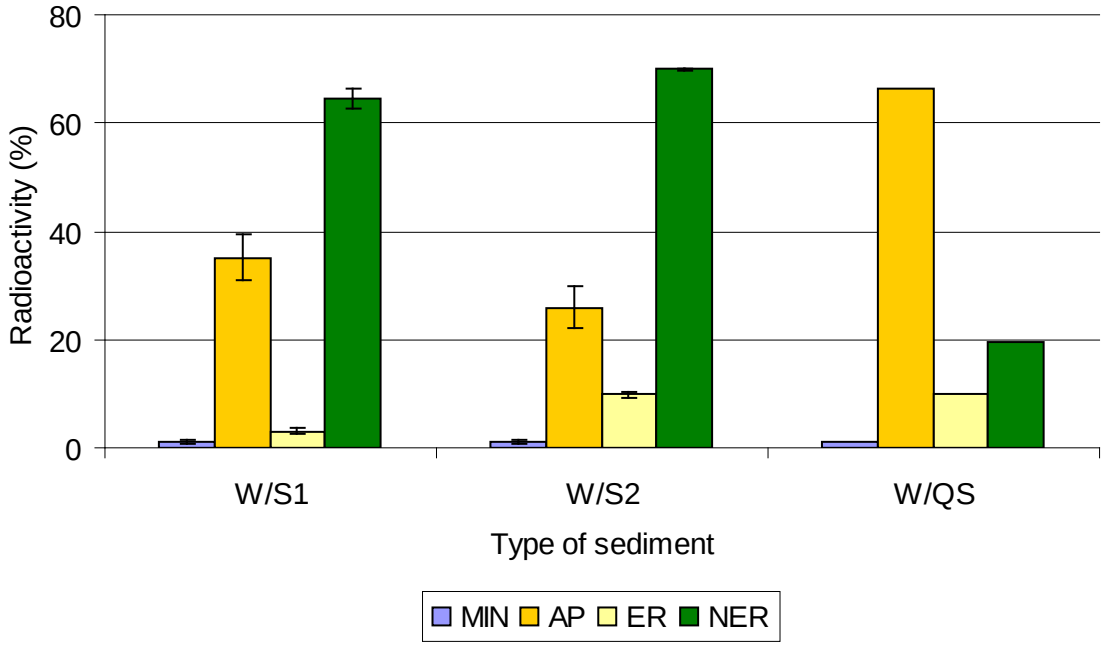


Figure 24: Balances of the transformation tests of ^{14}C-diclofenac in different water/sediment systems after 10 h/d irradiation for 3 days using laboratory batch system.

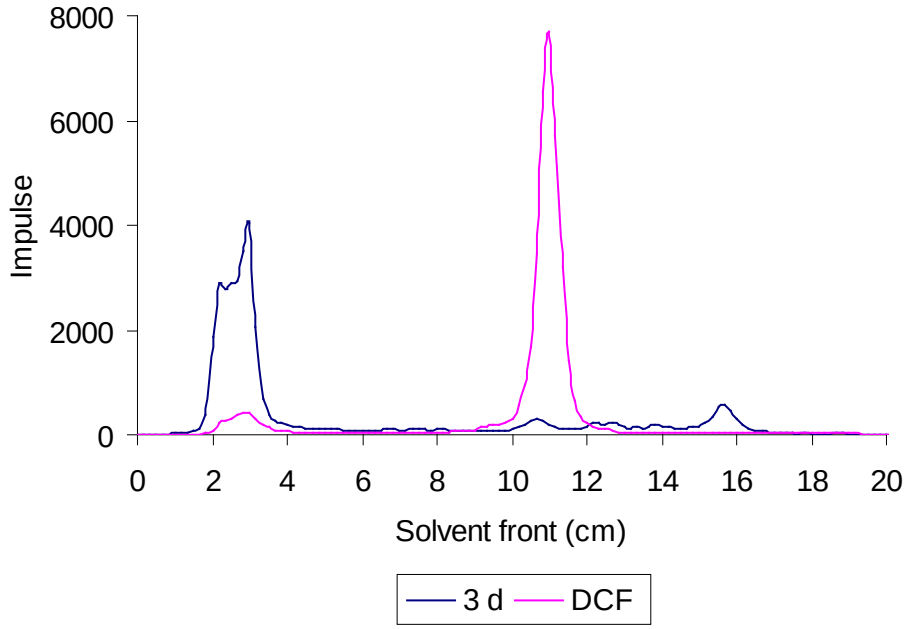


Figure 25: RTLC chromatogram after irradiation of ^{14}C-diclofenac in the water phase of WS1 for 3 days, extracted by SPE and developed by first solvent (n-hexane/ethyl acetate/acetic acid; 50:50:1 v/v/v)

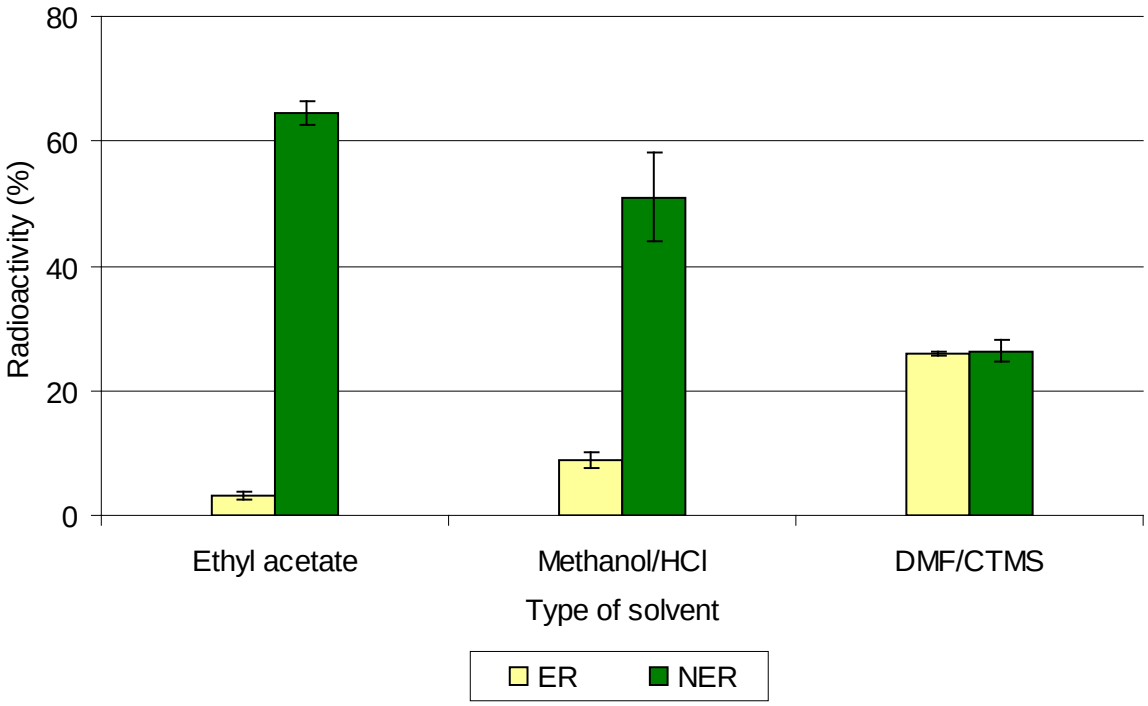
4.4 Chemical characterization of non-extractable residues

In the irradiation experiment, the rapid disappearance of diclofenac in both sediments under the laboratory conditions was mainly attributed to its photosensitivity resulting in the formation of several unknown highly reactive intermediate products which were assumed to be the reason of the formation of high amounts of the non-extractable residues. In case of WS1 sediment 65 ± 2 % was defined as non-extractable residues by the ethyl acetate extraction step. To check the extraction efficiency of this step, the extraction power was enhanced using methanol/hydrochloric acid. Only 7 ± 0.7 % were additionally extracted while 51 ± 5 % remained again non-extractable. For derivatization of the functional groups of the sediment identified as a sink for non-extractable residues [Heise et al., 2006, Haider et al.,1992], silylation technique was applied as a third step of the sequential extraction procedure. After silylation, 26 ± 0.2 % were then released while 26 ± 1 % remained as non-extractable residue **[Figure 26]**. The complete data set are listed in **Appendix, Table A28**.

Since radioactivity could be sequentially extracted, the bioavailability of these unknown transformation products onto the sediment could be also expected. In this case, sediments could not only play an important role as a sink of the transformation products but also as source due to the remobilization process. During the sequential extraction processes, furthermore, the extracts obtained after each step were screened for diclofenac by RTLC. Additionally, the ethyl acetate extracted sediments obtained after incubation for 100 d in the dark were extracted sequentially and screened by the same way. As shown by RTLC, diclofenac could not be detected in any extracts indicating that only its transformation products were able to interact strongly with the sediments.

4.5 Role of sediment in elimination of phototransformation of diclofenac

It is obvious that the role of sediment as a sink for the transformation products of diclofenac can not be neglected. In contrast to water/sediment systems, therefore, irradiation experiments were performed using native and demineralized water, without sediments, for estimation of this role. After 3 days irradiation period, the radioactivity amounts recovered were 92 and 107 % for native and demineralized water, respectively. In comparison with the radioactivity amounts recovered from the water phase of WS1 (almost 35 %), the sediment eliminated more than 55 % and 70 % of the phototransformation products formed in the water phases of native and demineralized water, respectively. At maximum, 3.5 % of the initially applied radioactivity could be detected by RTLC as ^{14}C-diclofenac in the water phase at the termination of the experiments.



DMF = dimethylformamide, CTMS = chlorotrimethylsilane, HCl = Hydrochloric acid

Figure 26: Chemical characterization of the non-extractable residues of sediment WS1 by means of sequential extraction technique after the 3-d irradiation experiment

4.6 GC/MS analysis

4.6.1 Fate of diclofenac after irradiation in pure water

GC/MS is one of the most frequently used tools for identifying transformation products. Two important advantages of GC/MS methods are known. First, the large amount of structural information that can be yield by the full scan mass spectra obtained under electron impact (EI) ionization. Second, the possibility of using commercial libraries makes the identification of unknowns feasible. However, GC/MS has important drawbacks because of its limited capability for analyzing very polar, less volatile, thermolabile compounds in addition to the long run times and the sample preparation time in case of derivatization of polar compounds. As a polar compound, diclofenac has to be derivatized before GC/MS analysis for more sensitive detection. Several methods have been described in the literature for derivatization of diclofenac. In the present study, diclofenac was derivatized according to Moeder et al. (2007) who described the derivatization procedure using pentafluorobenzyl bromide in aqueous potassium carbonate solution. After heating the reaction mixture for 1 h at 60 °C, they extracted the derivatized compound with n-hexane which was finally concentrated to 200 µL before GC/MS analysis in negative chemical ionization mode.

As a result of following this derivatization method, three peaks were observed in the total ion current chromatogram of the present study. By the interpretation of the mass spectra, these peaks could be identified as the intramolecular cyclization product of diclofenac [1-(2,6-dichlorophenyl)indolin-2-one] (m/z 277) which known as diclofenac artifact, diclofenac-pentafluorobenzylester (m/z 475), and, the derivatized diclofenac artifact, 1-(2,6-dichlorophenyl)indolin-2-one-pentafluorobenzyl ester (m/z 456) **[Figure 27]**. The latter one was found to be the most abundant substance which could be formed as a result of loss of water and an additional proton abstraction from the intact diclofenac-pentafluorobenzylester derivative. The occurrence of these compounds together in the same run could be only explained by an incomplete derivatization reaction or contamination of the diclofenac standard solution. However, the latter was excluded by means of HPLC/UVD purity test detecting no signal for presence of impurities where the standard solution of diclofenac was detected as a definite single signal **[Figure 28]**.

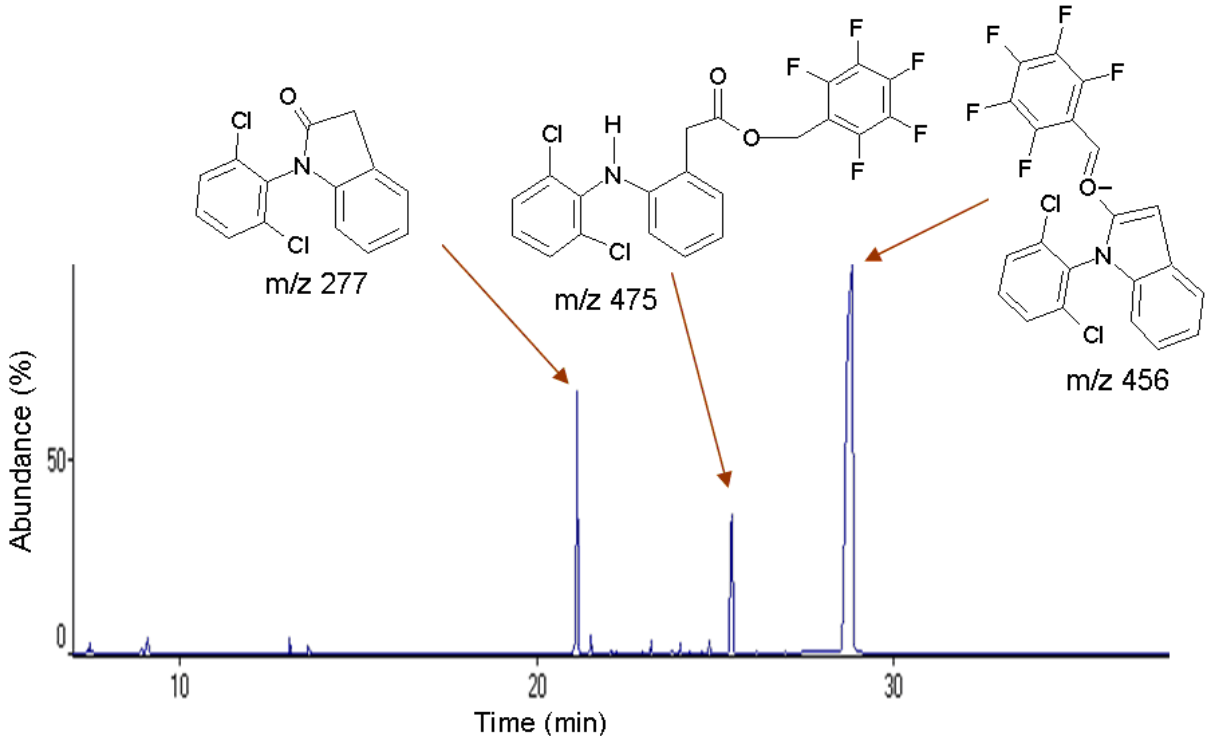


Figure 27: **Total ion current chromatogram of derivatized standard solution of diclofenac detected by GC/MS in full scan (EI) mode before optimization of the derivatization conditions**

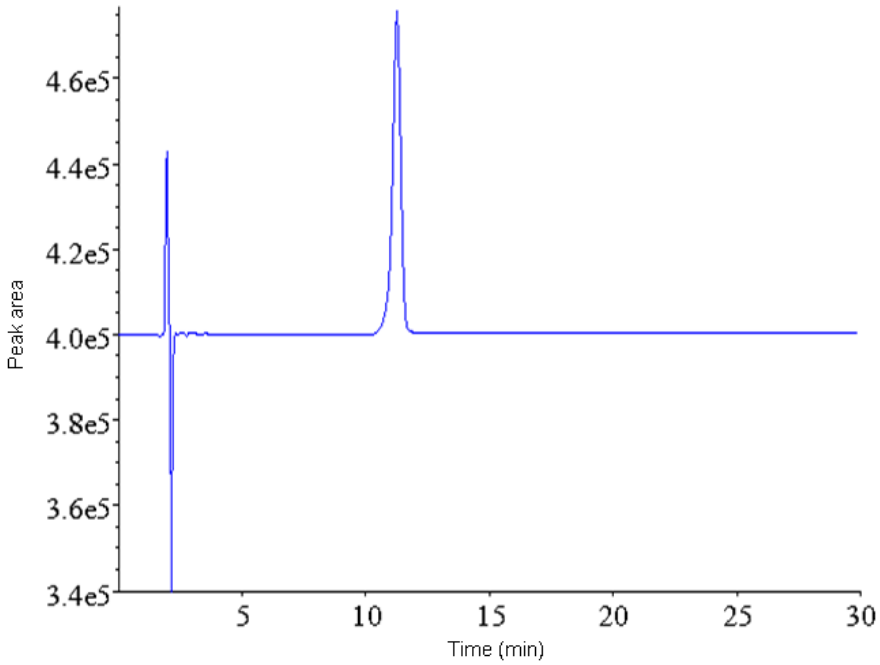


Figure 28: **Purity test of diclofenac by means of HPLC**

For quantitation purposes, therefore, different factors affecting the derivatization reaction were examined to optimize the derivatization conditions in order to obtain single derivatized compound such as volumes of pentafluorobenzyl bromide, reaction mixture, n-hexane and water as well as the heating time. As neutralizing agents, triethylamine or potassium carbonate were tested. The latter was additionally applied as a solution or as a powder. Under the optimized reaction conditions, only the pentafluorobenzylester of 1-(2,6-dichloro-phenyl)indolin-2-one with the molecular ion of m/z 456 was detected **[Figure 29]**. The reason of the disappearance of diclofenac-pentafluorobenzylester (m/z 475) and presence of derivatized diclofenac artifact instead is unknown. However, one of the most important advantages of the optimized derivatization was the evaporation of the reaction mixture up to dryness in order to remove any trace of water. Hence, to omit this important step may give the chance to the external water molecules to interact with the derivatized compound leading to the formation of both ions m/z 475 and m/z 456. On the other hand Moeder et al. (2007) proposed that, under the boundary conditions of the negative chemical ionization technique, this artifact formation was caused by a cyclization process under the intramolecular elimination of water followed by a proton abstraction from the diclofenac-pentafluorobenzylester resulting in the loss of 19 amu. This reason can be absolutely excluded since the present study was performed using the electron impact ionization technique.

Since the derivatized compound is not commercially available as standards, it was not possible to calculate the derivatization efficiency. However, since no by-product of derivatization were found to be present during the analysis, derivatization assumed to be complete. The stability of the derivative was checked for 3 days using its calibration curves. In this case the GC/MS was operated in the SIM mode for the molecular ion m/z 456. The results showed that even under the storage conditions at - 20 °C in the dark the derivative was not stable **[Figure 30]**. Therefore, derivatization was excluded for further investigation of diclofenac. In order to check for the photoinduced disappearance of diclofenac by GC/MS, the ethyl acetate extracts of the irradiated water samples were screened directly (without derivatization) for the cyclization product m/z 277 (artifact) as an indicator for the presence of diclofenac. The results showed that diclofenac was gradually transformed in the first five hours **[Figure 31]** and completely disappeared after one day irradiation period. The complete data set are listed in **Appendix, Table A29**.

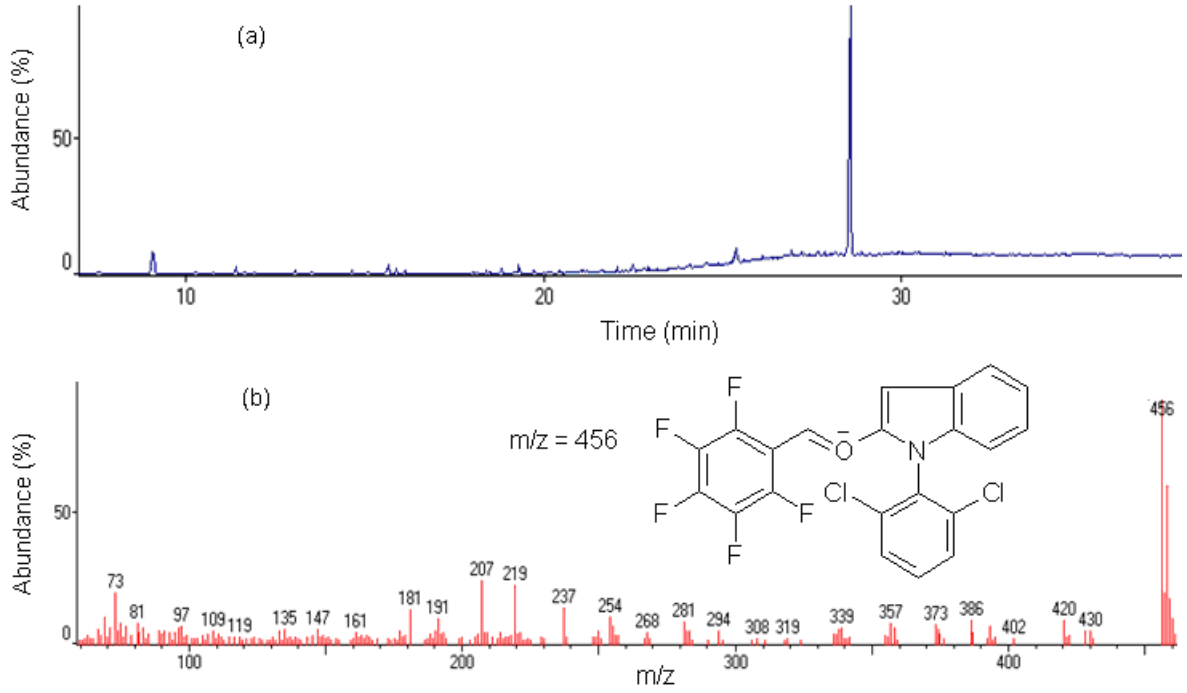


Figure 29: **(a) Total ion current chromatogram of the derivatized standard of diclofenac recorded by GC/MS applying electron impact ionization in full scan mode after optimization of the pentafluorobenzylation and (b) its corresponding mass spectrum**

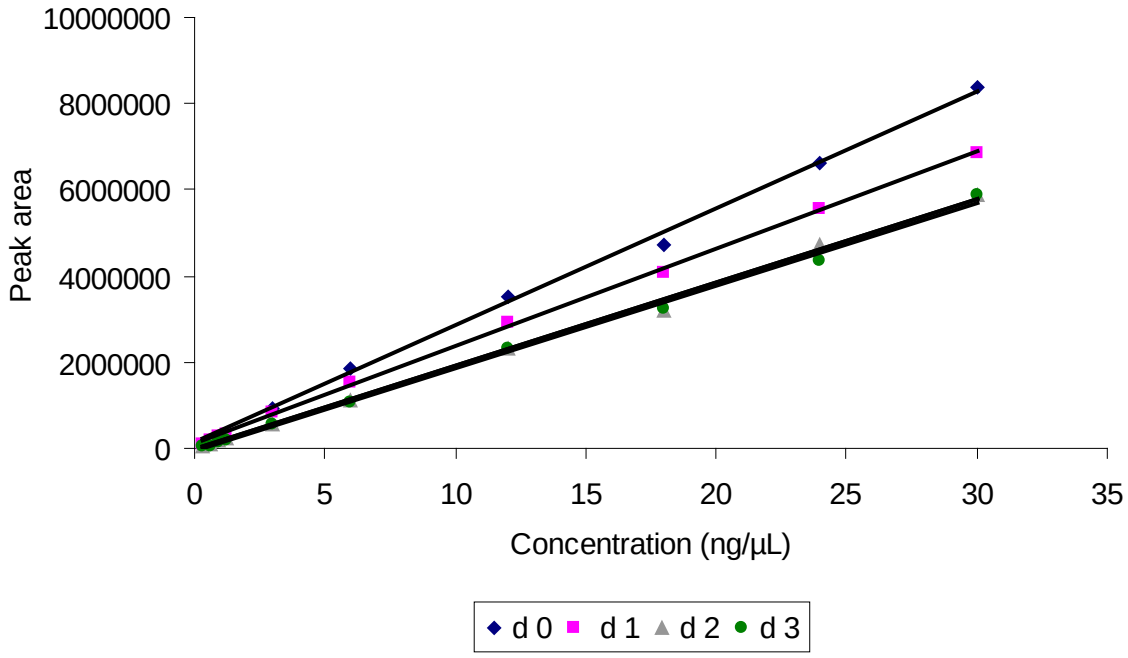


Figure 30: **Calibration curves of 1-(2,6-dichlorophenyl)indolin-2-one pentafluorobenzylester measured up to 3 days by GC/MS**

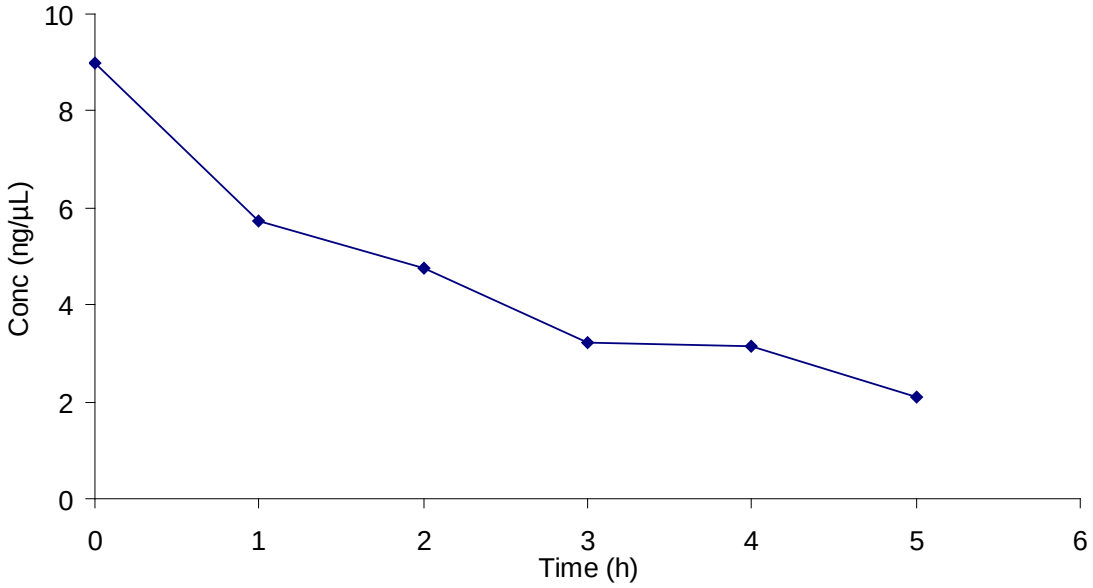


Figure 31: Dissipation of diclofenac after exposure to the light of the irradiation apparatus for different intervals in pure water detected by GC/MS

4.6.2 Origin of diclofenac artifact formation

Because of the loss of water, diclofenac can undergo an intramolecular cyclization resulting in formation of 1-(2,6-dichlorophenyl)indolin-2-one (molecular formula: $C_{14}H_9Cl_2NO$, molecular weight: 277) which is known as the artifact of diclofenac. The structure of this compound is shown in **Figure 32**.

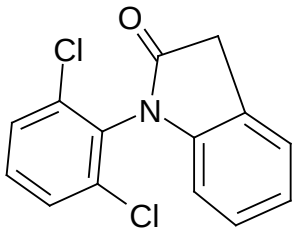


Figure 32: Structure of the artifact of diclofenac, 1-(2,6-dichlorophenyl)indolin-2-one

Numerous contradictory data have been published regarding the reason of the artifact formation during the GC/MS analysis of diclofenac. In the present study, therefore, a diclofenac standard solution in acetone was directly injected into the split/splitless injector of the GC/MS system at 280 °C in order to determine the exact origin of this indolinone cyclic amide structure. The characteristic full scan spectrum **[Figure 33]** depicting the molecular ion m/z 277 definitely confirmed that 1-(2,6-dichlorophenyl)-indoline-2-one was formed as a thermal artifact.

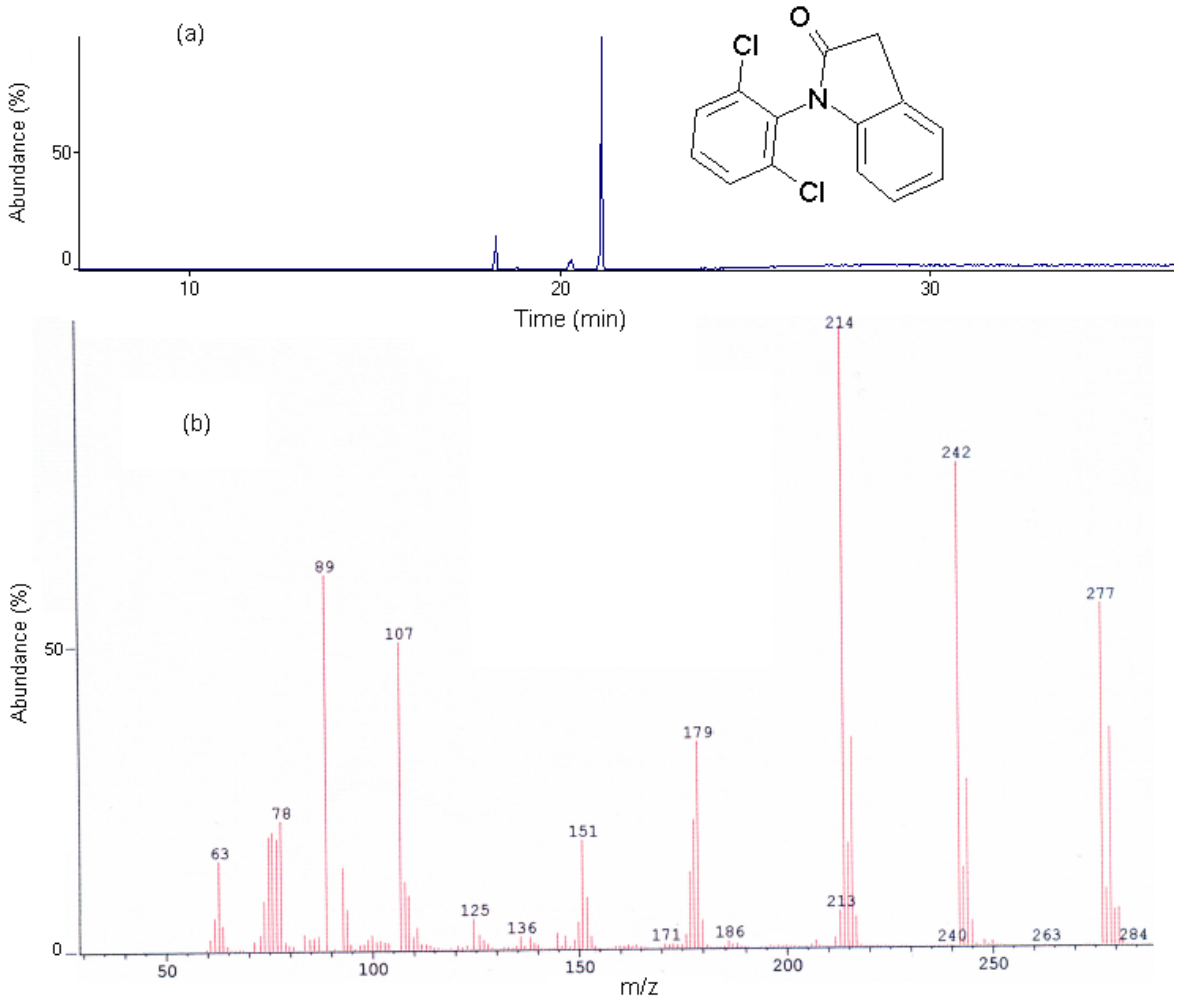


Figure 33: (a) Total ion current chromatogram and (b) mass spectrum of the artifact of diclofenac as detected by GC-MS in full scan (EI) mode

This reason was in agreement with some authors while others attributed the formation of artifact to different reasons such as acidic treatment, alkylation in alkaline media, MS conditions, sonolysis, or exposure to light. Others have identified the artifact of diclofenac as one of its bio- or phototransformation products. Under acidic conditions using 0.5 % sulphuric acid in 2.2.2-trifluoroethanol, Geiger et al. (1975) converted diclofenac into its artifact in order to be able to determine it in the biological fluids by GC/ECD. During biotransformation study of diclofenac in animal and man, Stierlin et al. (1979) detected several indolinone derivatives assuming that some of them were formed due to the acidic treatment of the urine samples and the others were formed pyrolytically at 180 °C in the ion source during the MS analysis as a result of thermal decomposition. Using GC/MS, Schneider and Degen, (1981) have published several indolinone derivatives formed after extractive alkylation of diclofenac under alkaline conditions. Although Palomo et al. (1999) excluded the acidic condition to be the

reason of the artifact formation as confirmed by IR analysis of acidified diclofenac solution (0.2 M HCl, pH 1.3), Reddersen and Heberer (2003) pointed to the SPE under acidic as the reason for artifact formation.

Furthermore, the behavior of diclofenac, in different solvent without derivatization, toward the temperature conditions used in the GC/MS was studied by El Haj et al. (1999). They found that, in methanol, diclofenac could be detected as methyl ester (stable major product) and indolinone cyclic structure (minor product). However, only the indolinone cyclic structure could be detected when diclofenac was dissolved in ethyl acetate. The high temperature of the GC injection port and/or the column is expected to be the reason of the esterification and cyclization which occur in the vapor state. Cyclization upon heating of γ and δ amino acids to give stable five or six membered ring has been already puplished [El Haj et al., 1999, Morrison and Boyd, 1987]. Additionally, the formation of the indolinone cyclic structure, after heating of diclofenac in acidified water at 100 °C for 90 min, was identified by GC/MS spectrum as a single peak and by TLC as a single spot (El Haj et al., 1999). They differentiated between diclofenac and the indolinone cyclic structure by measuring their UV absorption spectra. The latter UV spectra showed absorption maxima at 244 nm in aqueous acid and 275 and 303 nm in aqueous alkaline solution. Zajac et al. (1998) reported the formation of artifact as a result of heating process of diclofenac. Tudja et al. (2001) studied the thermal behavior of diclofenac sodium. They suggested the occurrence of an intramolecular cyclization reaction between amino and carboxylic group in solid diclofenac samples heated up to 270 °C resulting in the formation of the artifact. Additionally, sterilization (at 123 °C) of diclofenac by means of autoclaving was noted to form the artifact [Roy et al., 2001]. The mechanism of this reaction is shown in **Figure 34**.

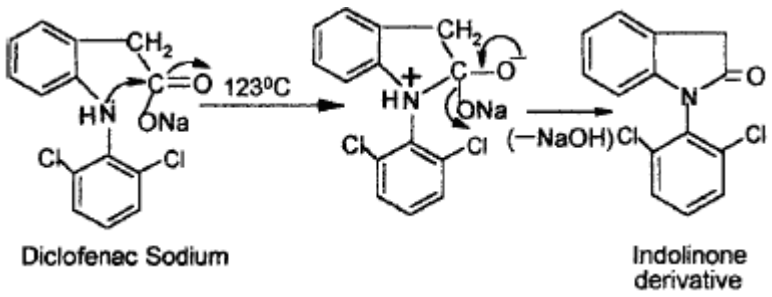


Figure 34: Formation of the indolinone artifact from diclofenac sodium [Roy et al., 2001]

Comparing diclofenac control samples analyzed by GC or LC, Kosjek et al. (2009) confirmed that the formation of the artifact was due to the thermal decomposition process. Sonolysis of a diclofenac solution has been reported as another reason for its formation [Hartmann et al., 2008]. Using LC/MS/MS/ESI, Giordano et al. (2003) identified the artifact when diclofenac either exposed to high temperature (160 °C) or exposed to light (60 W tungsten lamp) in presence of oxidant mixture (ferrous sulphate and ascorbic acid) due to the existence of free OH radicals. It was also detected by LC/MS/MS/ESI as one of the degradation products of diclofenac in ophthalmic solutions stored for 9 weeks at 60 °C [Galmier et al., 2005] or as degradation product in topical emulgel after long storage [Hajkova et al., 2002]. Additionally, it has been described by Kosjek et al. (2007a, b) as a one of the biotransformation products of diclofenac identified by means of GC/MS in a pilot wastewater treatment plant model study, by Bartels and von Tuempling Jr. (2007) as one of the major biotransformation products of diclofenac identified by means of GC/MS operating in electron impact ionization mode, by Moeder et al. (2007) due to the operation of GC/MS in negative chemical ionization mode and by Scheurell et al. (2009) as a phototransformation product formed in surface water. Furthermore, the 4'-hydroxy derivatives of the artifact was detected by means of LC/MS/MS in human urine samples as well as in WWTP effluent as a biotransformation product of diclofenac [Stuelten et al., 2008b]. Finally, according to European Pharmacopoeia (2005), the artifact can be formed as an intermediate impurity during the synthesis of diclofenac.

4.6.3 Detection of phototransformation products of diclofenac

For the identification of phototransformation products, the irradiated water samples were analyzed in two different ways by means of GC/MS in full scan mode. Hence, the extracts were either analyzed without derivatization for detection of possibly formed non-polar phototransformation products or after derivatization (as previously mentioned for diclofenac) for detection of possibly formed polar phototransformation products.

The results showed that phototransformation products could not be detected by GC/MS during those phototransformation tests. Despite pentafluorobenzylation, further signals could not be found after the 3-d incubation interval indicating that the initially applied parent compound and possibly formed phototransformation products were completely transformed under irradiation.

4.7 HPLC analysis

4.7.1 Detection and characterization of phototransformation products of diclofenac

In order to avoid any thermal stress during the flash evaporation in a GC split/splitless injector HPLC was applied. For this purpose, irradiation experiments were conducted for 5 h using a combined mixture of labeled and non-labeled diclofenac. During this time, samples were taken after an irradiation interval of 1 h. The sample extract of each interval were then screened by means of RTLC and additionally reversed-phase HPLC/UVD and HPLC/DAD for UV detectable phototransformation products (PTP) probably formed besides diclofenac.

To obtain detailed information about the phototransformation products during the experiment, the total radioactivity was measured in the different compartments through the liquid-liquid extraction procedure. The results were then used to setup the mass balances at the end of extraction step, to calculate the extraction efficiency of ethyl acetate after each interval. By means of this method, an idea about the chemical nature (polarity) of the phototransformation products could be obtained. The recovery and the percentage of the radioactivity in each compartment related to the initially applied radioactivity are listed in **Table 12**. It was found that the total radioactivity extracted by means of ethyl acetate gradually decreased from 93 to 56 % within 5 h irradiation period. Conversely, the total radioactivity remained in the water phase gradually increased from 12 to 41 % within the same irradiation period. In another way, the extraction efficiency of the ethyl acetate decreased from 86 to 53 % as the irradiation time increased from 1 to 5 h. The formation of more polar phototransformation products that could not be extracted by ethyl acetate during the irradiation experiment was the most suitable interpretation for these results.

Additionally, the extracts developed on normal phase TLC plates and screened by RTLC referred to presence of at least three phototransformation products beside the parent compound **[Figure 35]**. As it was expected, the radioactivity amount identified as diclofenac decreased from 72 to 26 % within the irradiation period. The elimination constant (K_{elim}) was found to be 0.2 per hour resulting in DT_{50} of 3 h. Furthermore, 3 obvious UV absorbent phototransformation products could be detected beside diclofenac by means of HPLC/UVD and HPLC/DAD at reasonable retention times **[Figure 36, 37]**. On the other hand, RTLC screened extracts developed on reversed phase TLC plates, did not show the same separation pattern obtained by HPLC. This might be attributed to the low separation performance of TLC when compared to HPLC.

Table 12: Balances of transformation tests of ^{14}C-diclofenac in pure water after irradiation for different intervals during the liquid-liquid extraction steps

Irradiation interval (h)	(a)	(b)	(c)	(d)	(e)
1	108	12	93	86	105
2	108	13	86	80	99
3	108	25	77	71	102
4	108	38	64	60	102
5	106	41	56	53	97

(a) Recovery percentage of the radioactivity in the 100 mL water sample directly before the liquid-liquid extraction.

(b) Recovery percentage of the radioactivity remained in the 100 mL water sample after the liquid-liquid extraction.

(c) Recovery percentage of the radioactivity extracted by ethyl acetate after the liquid-liquid extraction.

(d) Extraction efficiency percentage of ethyl acetate (C/A x 100)

(e) Mass balance percentage of the radioactivity in the water phases and in the ethyl acetate extracts (B+C)

4.7.2 Dissipation behavior of diclofenac and its phototransformation products

Aiming to obtain complete transformation of diclofenac, the irradiation intervals were expanded up to 10 h. The concentrated extracts were screened by HPLC/UVD. It was obvious that the peak areas of diclofenac and PTP1 decreased the longer the irradiation time was while the peak areas of PTP2 and PTP3 remained nearly constant **[Figure 38]**. The concentration of diclofenac dropped rapidly from 100 ng/µL to 33 ng/µL within the first 2 h and then to 4 ng/µL after 10 h of irradiation. The complete data set are listed in **Appendix, Table A30**. Furthermore, the direct HPLC/UVD analysis of the aqueous aliquots taken before the liquid/liquid extraction step confirmed that neither diclofenac nor related PTPs remained in the water samples after extraction procedure. To exclude dilution effect as a reason for this observation, the water remained after liquid/liquid extraction was further extracted by means of SPE confirming the previous results. However, in the previous radiotracer test, considerable radioactivity amount could be detected in the water phase after liquid/liquid extraction process. Therefore, it can be assumed that not only diclofenac but also its phototransformation products were photosensitive and hence further transformation for the

detected phototransformation products might be expected. This assumption was confirmed from their absorption spectra in which the peak tail of the detected phototransformation products expanded over 300 nm **[Figure 39]**. Thus, the last part of their absorption bands overlap with the beginning of the wavelengths emitted by the radiation source (cut off 290 nm) used in the present study.

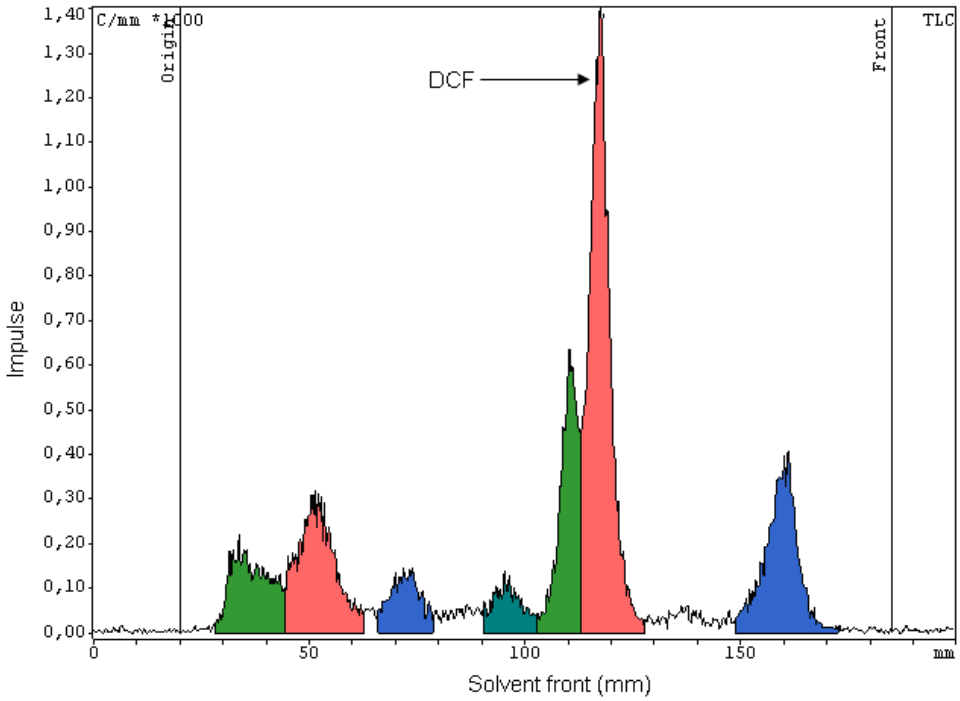


Figure 35: **RTLC chromatogram of a transformation test of ^{14}C-diclofenac in pure water after irradiation for 5 h, extracted by liquid/liquid extraction and developed using n-hexane/ethyl acetate/acetic acid (50:50:1 v/v/v) on normal phase TLC plate**

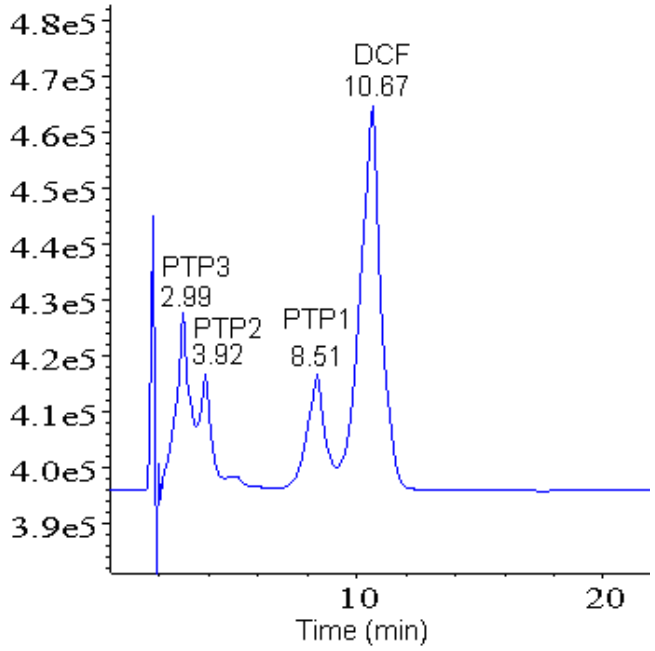


Figure 36: **Chromatogram of a transformation test of diclofenac after irradiation for 5 h in pure water as detected by HPLC/UVD at λ = 280 using methanol/water/ phosphoric acid (70: 30: 0.1, v/v/v) as mobile phase**

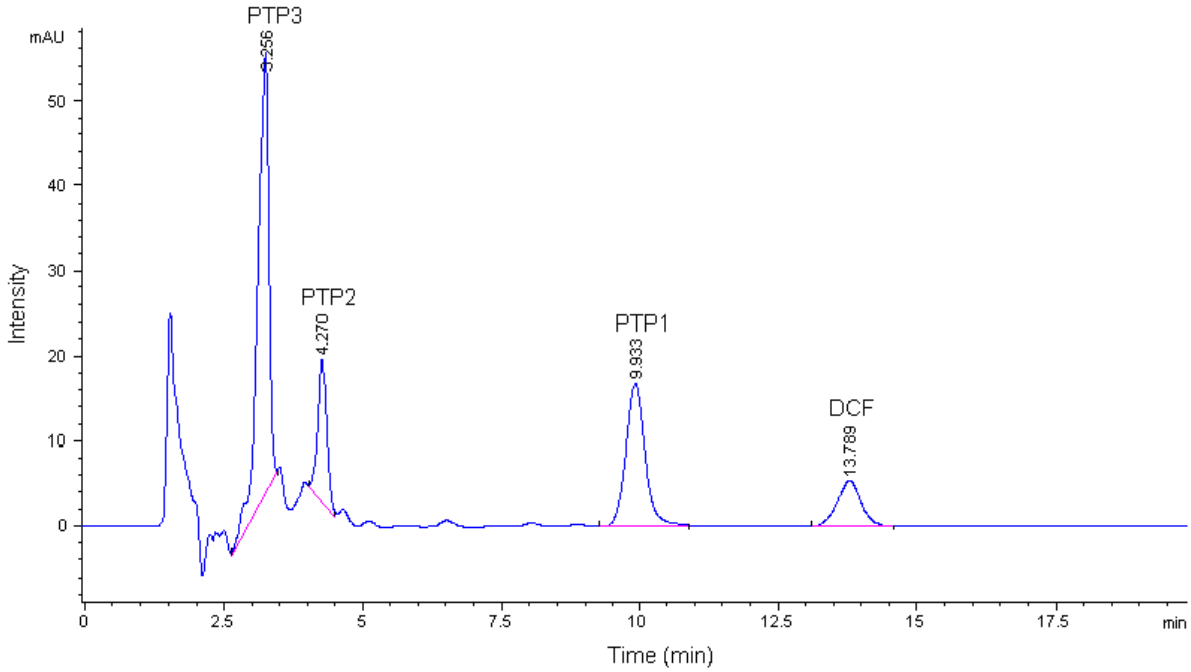


Figure 37: Chromatogram of a transformation test of diclofenac after irradiation for 5 h in pure water as detected by HPLC/DAD at λ = 242 nm using acetonitrile/water/formic acid (50: 50: 0.1, v/v/v) as mobile phase

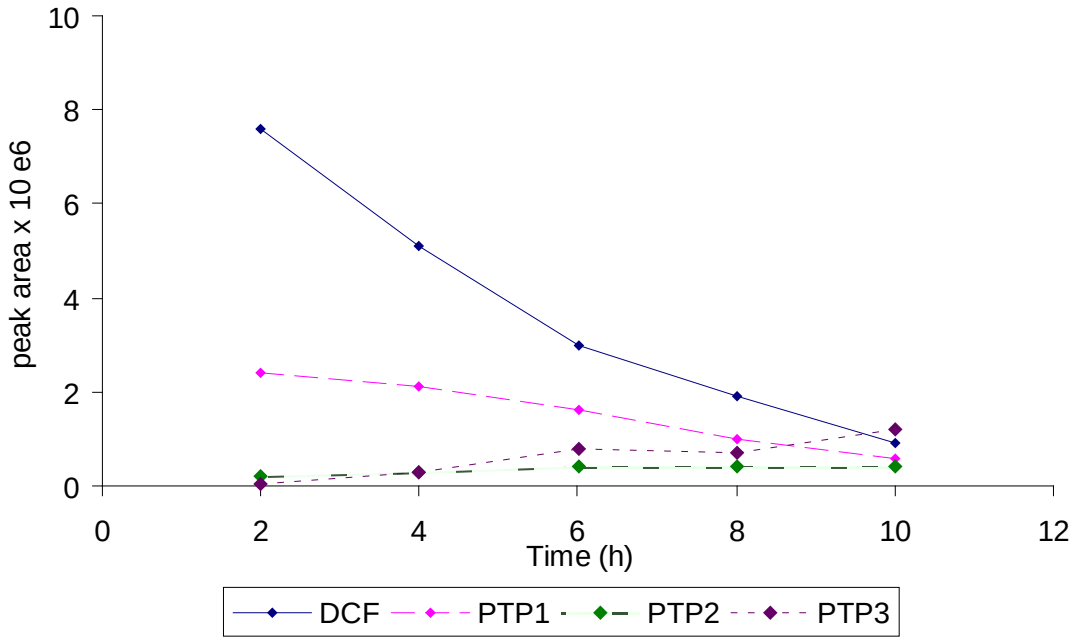
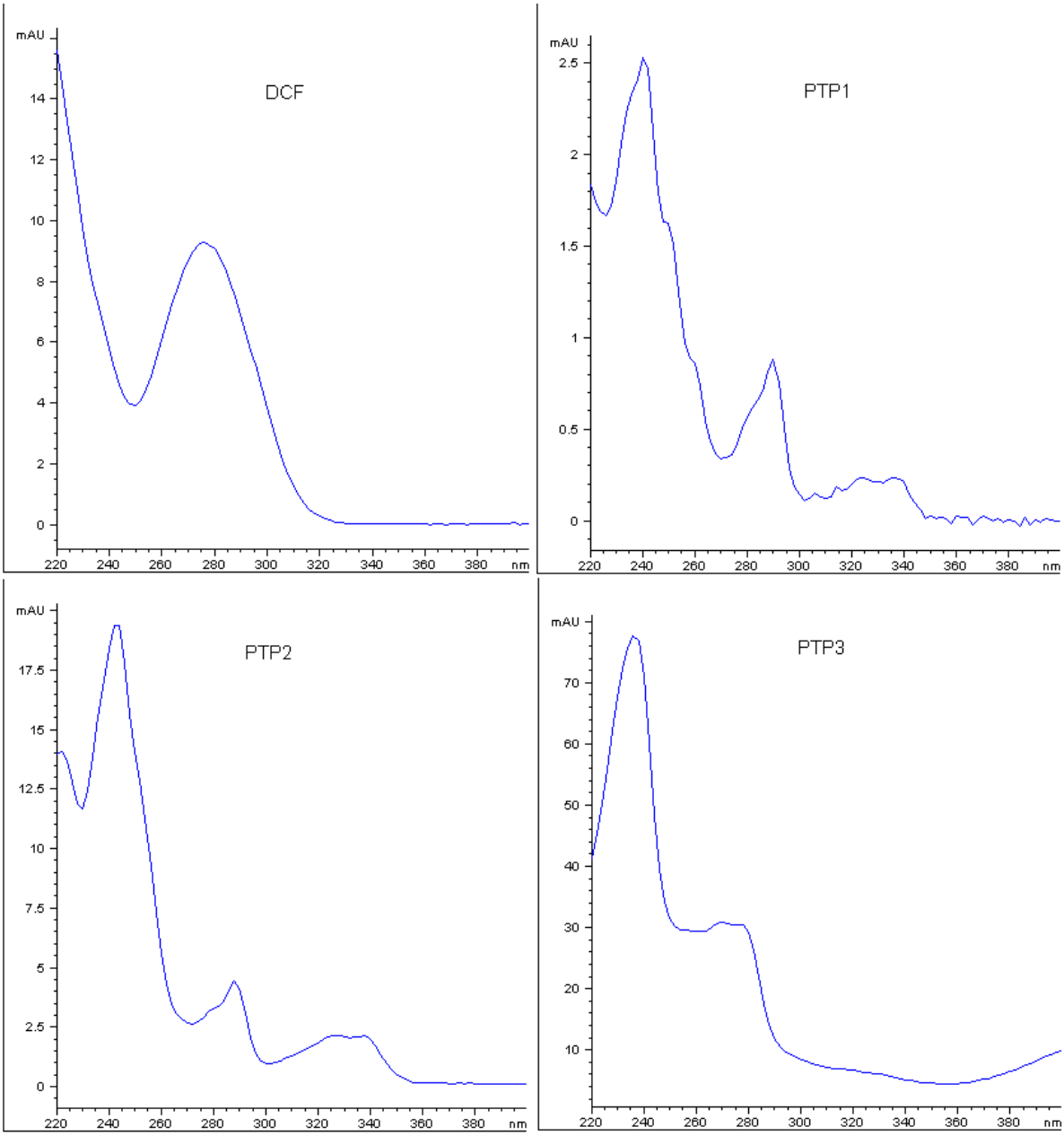


Figure 38: Peak areas of diclofenac and its PTPs after exposure of diclofenac to irradiation in pure water for different intervals as detected by HPLC/UVD at λ = 254 nm using acetonitrile/water/formic acid (50: 50: 0.1, v/v/v) as mobile phase



PTP = phototransformation product

Figure 39: UV absorption spectra of diclofenac and its PTPs detected by HPLC/DAD after irradiation of diclofenac in pure water for 5 h

4.8 LC/MS/MS analysis

4.8.1 Method optimization

Preliminary, several parameters had to be optimized in order to find the best boundary conditions for LC/MS/MS analysis. These parameters were optimized for diclofenac, since its phototransformation products (PTPs) were not available as reference standards. Basically, there are two types of parameters, i.e., compound dependent parameters and flow dependent parameters that can be adjusted manually or automatically. In the present study, these parameters were adjusted automatically **[Figure 40]**.

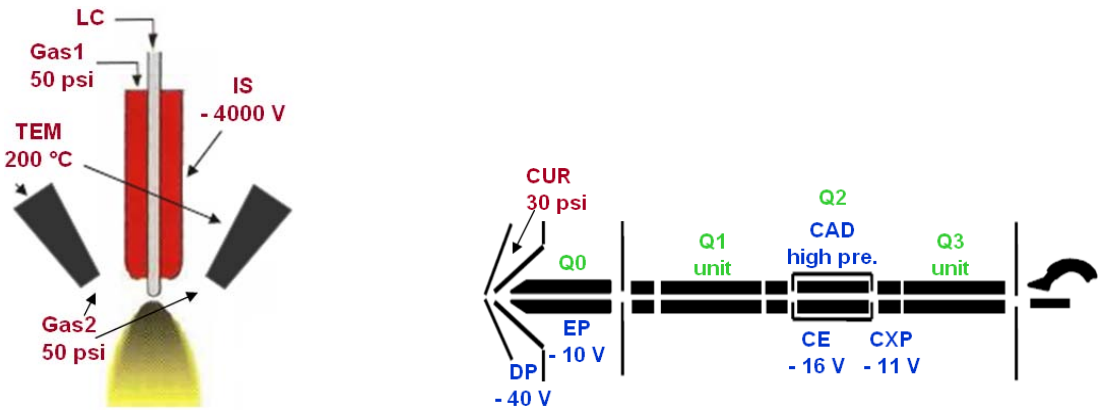


Figure 40: (a) Flow dependent parameters and (b) compound dependent parameters and their optimum values for diclofenac

Other parameter such as mode of operation and scan type had to be adjusted manually. For this purpose, 10 ng/µL of diclofenac were tested for the compound dependent parameters in both positive and negative operation mode using $[M+H]^+$ (m/z 296) and $[M–H]^-$ (m/z 294), respectively. The $[M–H]^-$ ion recorded was of definite higher intensity than the $[M+H]^+$ ion was. Therefore, the negative mode was selected for the further study. Additionally, different scan types were applied in order to obtain appropriate structure-specific information without interfering noise peaks. For this purpose, full scan using the first quadrupole (Q1 scan), the third quadrupole in the linear ion trap mode (EMS scan) to increase the sensitivity by EMS or a combination between EMS and EPI scan types using IDA method were tried. The latter was found to be the most appropriate one because the gained mass specific information that could be used for selection of the product ion with the most abundant mass to charge ratio for further LC/MS/MS experiments. Furthermore, different gradient and isocratic organic mobile phases were tested **[Table 13a, b, c]** in order to achieve better resolution, higher separation quality and proper peak shapes for diclofenac and its PTPs. The results showed

that the highest resolution was achieved by the isocratic mobile phase consisting of acetonitrile/ 0.5 % formic acid [50:50 (v/v)].

Table13: Mobile phases used in method optimization of diclofenac

(A)

Total time (min)	Flow rate (µL/min)	Eluent A % 0.1 % acetic acid in LC/MS water	Eluent B % 0.1 % acetic acid in methanol
0.00	300	35.0	65.0
10.00	300	28.0	72.0
15.00	300	0.0	100
16.00	300	0.0	100
21.00	300	35.0	65.0
30.00	300	35.0	65.0

(B)

Total time (min)	Flow rate (µL/min)	Eluent A % 0.5 % formic acid in LC/MS water	Eluent B % Acetonitrile
0.00	300	90.0	10.0
15.00	300	10.0	90.0
20.00	300	10.0	90.0
21.00	300	90.0	10.0
26.00	300	90.0	10.0

(C)

Total time (min)	Flow rate (µL/min)	Eluent A % 0.5 % formic acid in LC/MS water	Eluent B % Acetonitrile
0.00	300	50	50
30	300	50	50

4.8.2 Identification of phototransformation products of diclofenac in water samples

After standard application, diclofenac was firstly irradiated in pure water instead of real water samples or water/sediments samples to avoid interference, contamination, signal suppression and falls positive finding that could hinder the identification of its phototransformation products due to matrix effects. For the identification purpose, the total ion current chromatograms obtained by LC/MS/MS analysis of irradiated diclofenac samples were comprehensively investigated for every peak like shape. As a result, only four peaks could be identified as diclofenac and its phototransformation products due to their specific mass spectra at certain retention times.

As an example, the total ion current chromatogram for the 2-h irradiation interval is given in **Figure 41** in which peaks regarding diclofenac and its phototransformation products, i.e., PTP1, 2 and 3 were pointed by means of arrows. Furthermore, EMS and EPI mass spectra of diclofenac and PTP1, 2 and 3 referred to the most abundant product ions of 250, 214, 196 and 185 m/z, respectively **[Figure 42-45]**. In order to find the precursor ions that were generating these product ions, the latter were individually subjected to the precursor ion scan mode. In each case, only one peak was observed in the total ion current chromatogram of this scan mode. Hence, each product ion was generated by means of only one precursor ion. As additionally shown by their related mass spectra m/z 294, 258, 240, and 229 were detected to be the precursors of diclofenac and PTP1, 2 and 3, respectively **[Figure 46-49]**. It was obvious that the precursor ions $[M-H]^-$ of diclofenac, PTP1, and PTP2 were characterized by the loss of 44 amu which exactly corresponding to the loss of $[-COO]^-$. Therefore, it can be concluded that the structures of these transformation products include presence of carboxylic groups. On the other hand, the ion at m/z 229 $[M-2H]^{2-}$ was characterized by the loss of 26 amu (C_2H_4) which is common for the fragmentation of the aromatic compounds followed by 18 amu (H_2O) which is common for the fragmentation of the alcoholic compounds.

As a diphenylamine compound, diclofenac is almost converted to carbazole derivatives when exposed to light [Bowen and Eland, 1963]. According to the m/z and the fragmentation pattern of the precursor ions, three carbazole derivatives could be identified as possible phototransformation products for diclofenac. These carbazole derivatives are 8-chloro-9H-carbazole-1- acetic acid (PTP1), 8-hydroxy-9H-carbazole-1- acetic acid (PTP2) and 8-chloro-9H-carbazole-1-methanol (PTP3). The structure of diclofenac and its phototransformation products are listed in **Table 14**. On the other hand, the other peaks investigated in the total ion current chromatogram of all intervals had nearly the same MS spectra as the base line

indicating that these peaks can be considered as noise peaks **[Figure 50]**. Therefore, the most relevant diclofenac phototransformation process is the photocyclization to the corresponding monochlorinated carbazole PTP1 and PTP3. Furthermore, the substitution of the chlorine atom by the hydroxyl group led to the formation of PTP2. Those phototransformation products were also described by Agueera et al. (2005) and Moore et al. (1990) by the interpretation of LC/TOF-MS and ^{1}H-NMR spectra, respectively. Within the present study, however, a dimer formation was not observed. The concentration of diclofenac was determined in MRM mode using the transition of m/z 294 to 250. It was observed that the amount of diclofenac dropped to 34 % and 4 % after 2 and 10 h of exposure to the light, respectively **[Figure 51]**. The complete data set are listed in **Appendix, Table A31**.

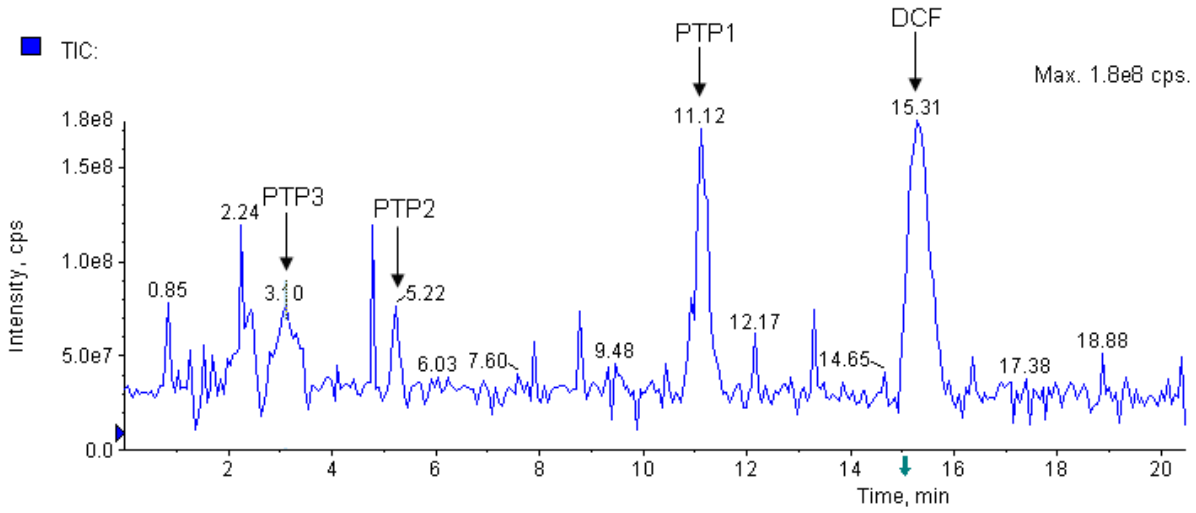


Figure 41: Total ion current chromatogram of pure water sample after 2-h irradiation showing the presence of 3 phototransformation products besides diclofenac as detected by LC/MS/MS

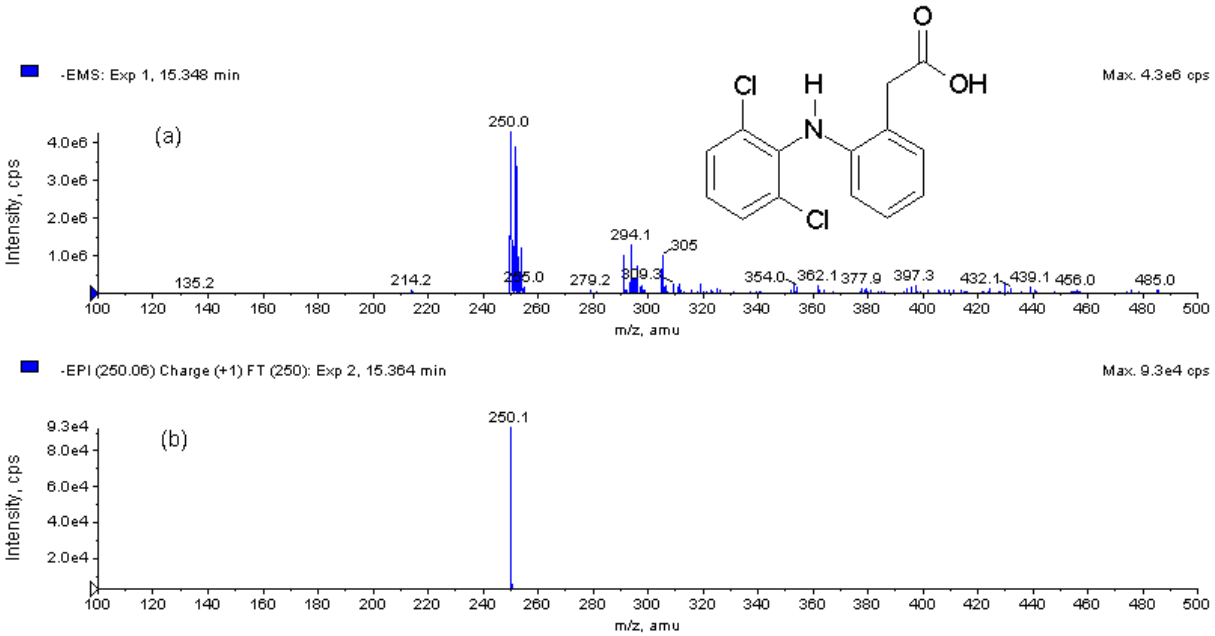


Figure 42: Mass spectra of diclofenac detected by LC/MS/MS using information depending acquisition method: (a) Enhanced full mass scan (EMS) and (b) enhanced product ion scan (EPI)

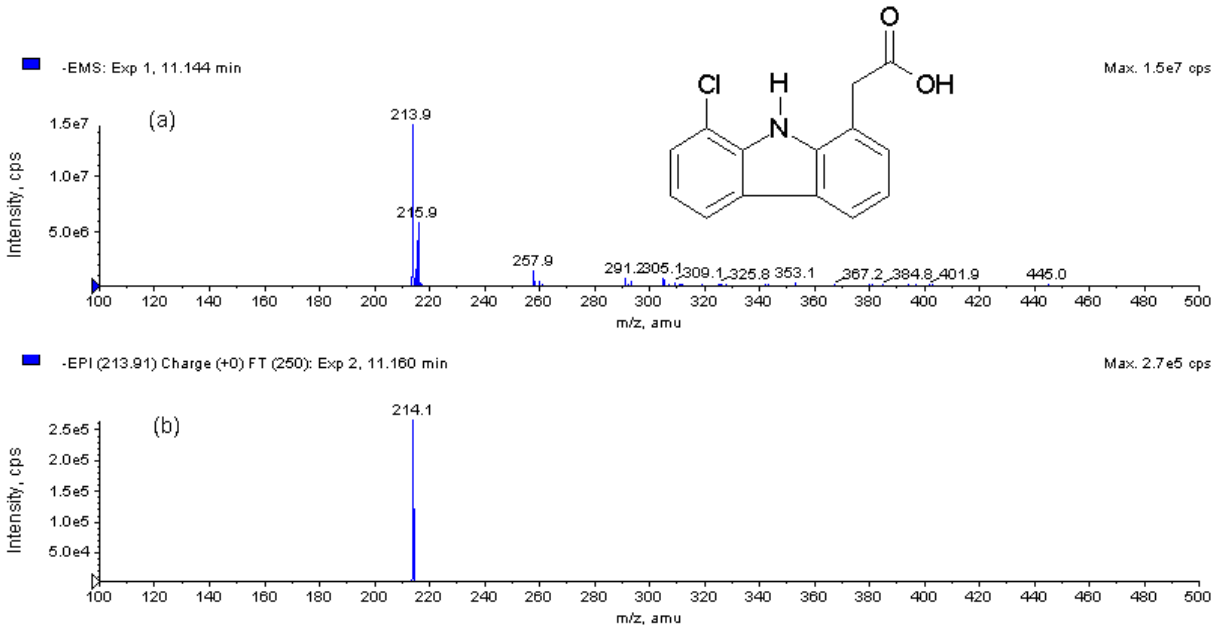


Figure 43: Mass spectra of 8-chloro-9H-carbazole-1-acetic acid detected by LC/MS/MS using information depending acquisition method: (a) Enhanced full mass scan (EMS) and (b) enhanced product ion scan (EPI)

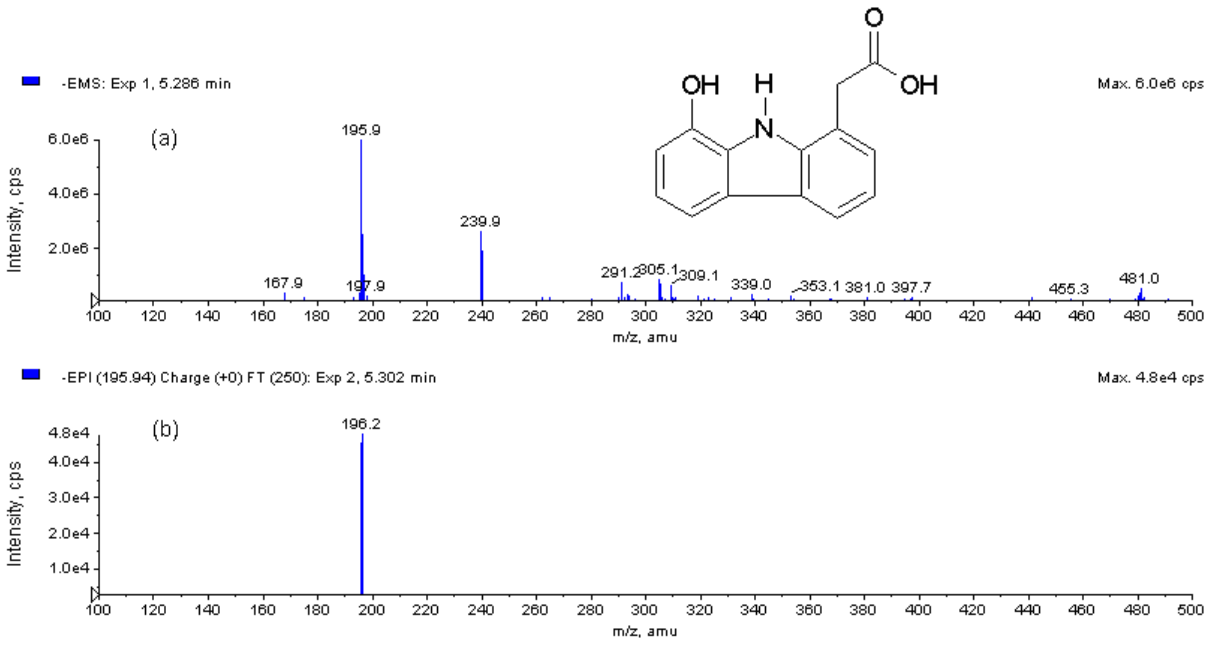


Figure 44: **Mass spectra of 8-hydroxy-9H-carbazole-1-acetic acid detected by LC/MS/MS using information depending acquisition method: (a) Enhanced full mass scan (EMS) and (b) enhanced product ion scan (EPI)**

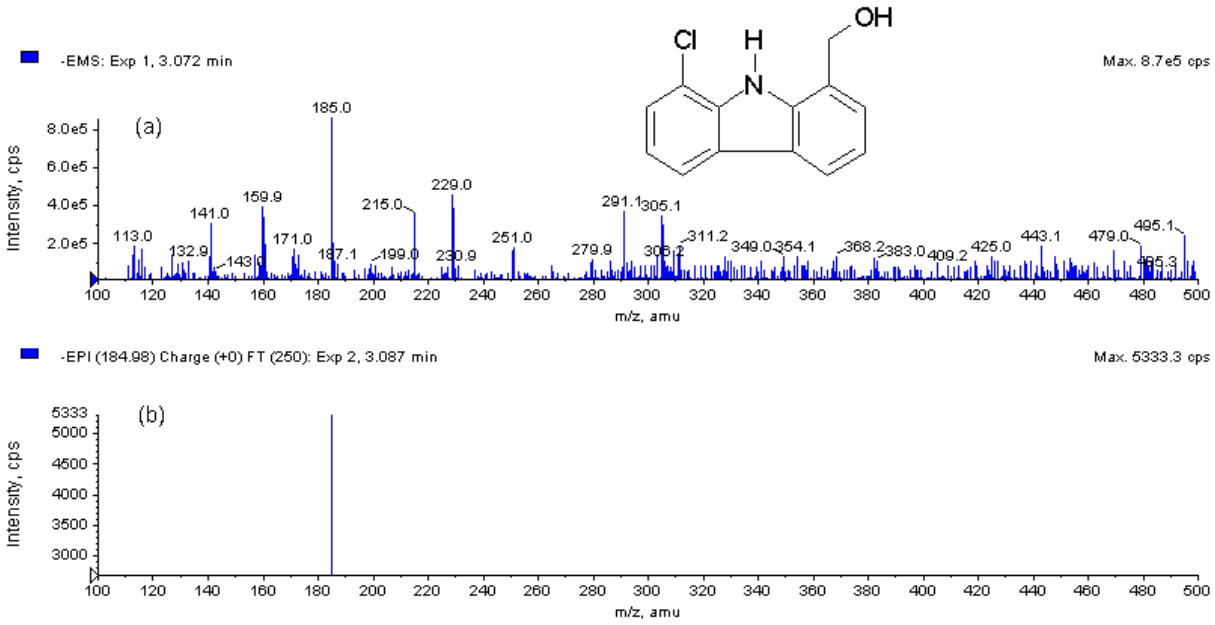


Figure 45: **Mass spectra of 8-chloro-9H-carbazole-1-methanol detected by LC/MS/MS using information depending acquisition method: (a) Enhanced full mass scan (EMS) and (b) enhanced product ion scan (EPI)**

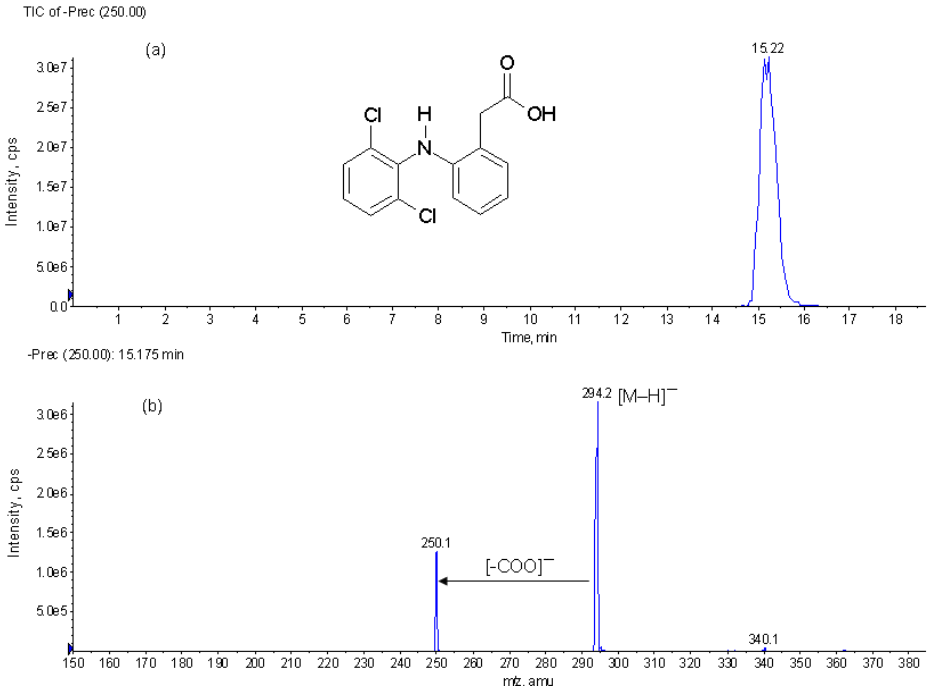


Figure 46: (**a) Precursor total ion current chromatogram of product ion 250 m/z detected by LC/MS/MS and (b) its corresponding MS spectrum identifying diclofenac (m/z 294) as the precursor ion**

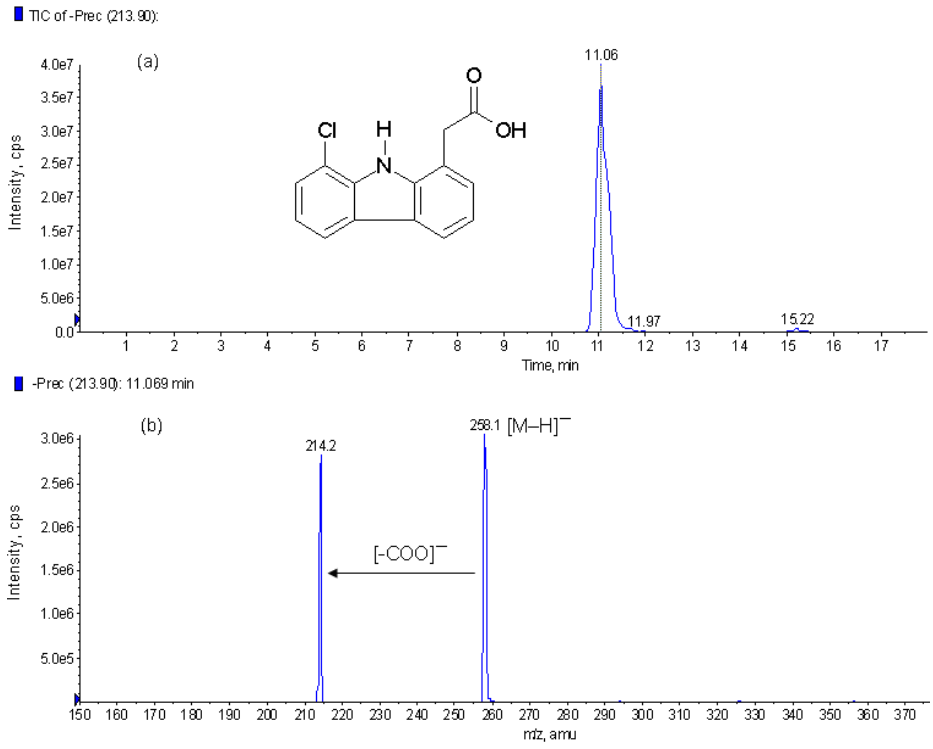


Figure 47: (a) Precursor total ion current chromatogram of product ion 214 m/z detected by LC/MS/MS and (b) its corresponding MS spectrum identifying 8-chloro-9H-carbazole-1-acetic acid (m/z 258) as the precursor ion.

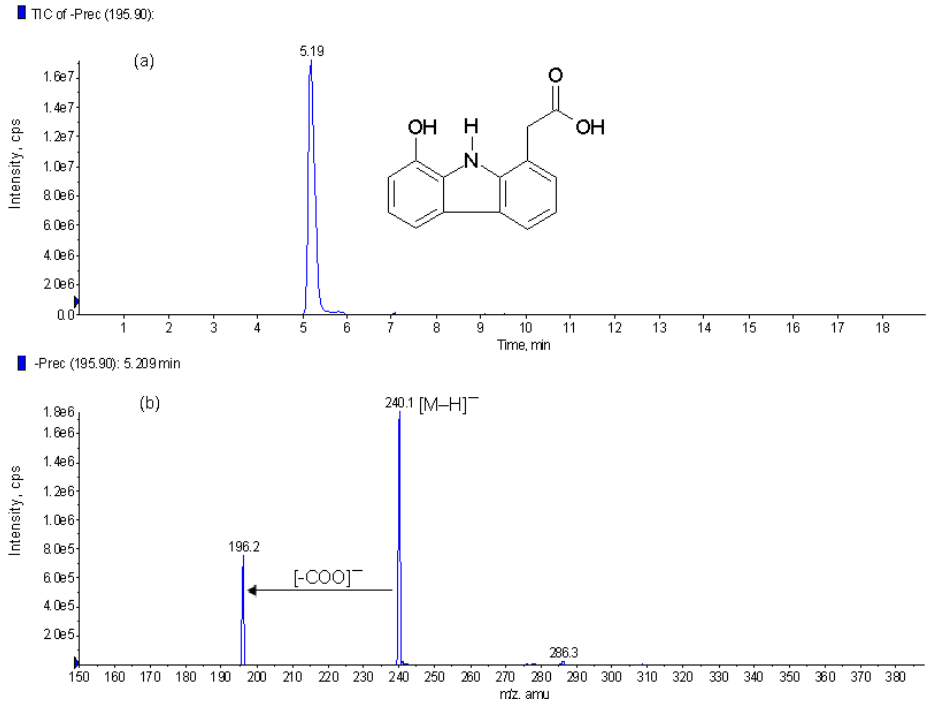


Figure 48: (a) Precursor total ion current chromatogram of product ion m/z 196 detected by LC/MS/MS and (b) its corresponding MS spectrum identifying 8-hydroxy-9H-carbazole-1- acetic acid (m/z 240) as the precursor ion

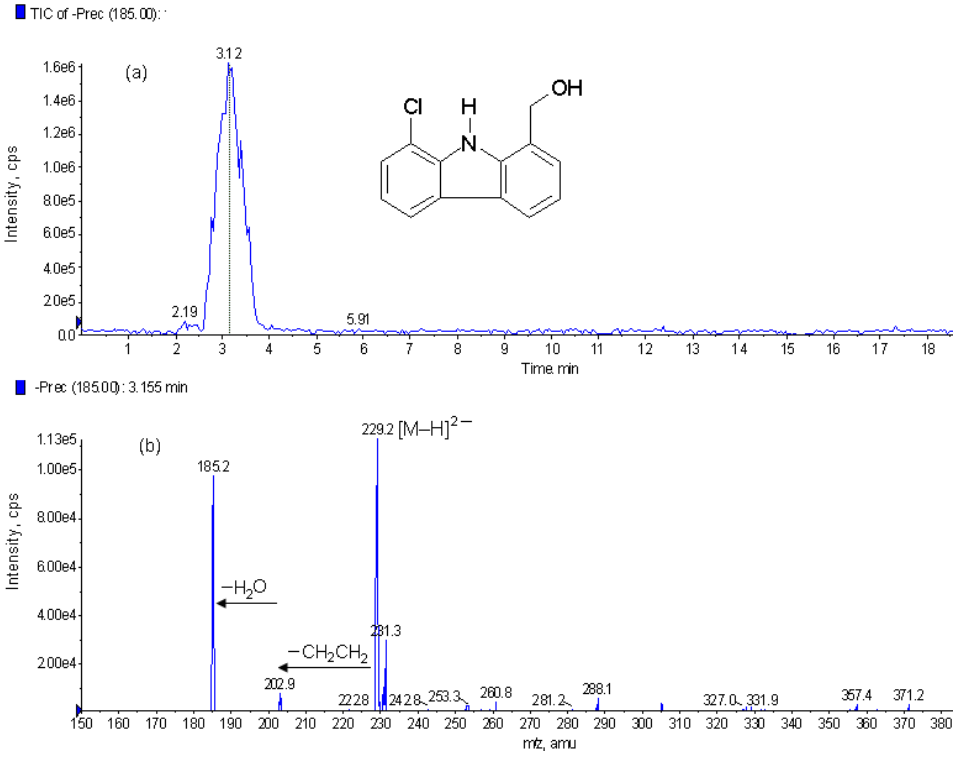


Figure 49: (a) Precursor total ion current chromatogram of product ion m/z 185 detected by LC/MS/MS and (b) its corresponding MS spectrum identifying 8-chloro-9H-carbazole-1-methanol (m/z 229) as the precursor ion

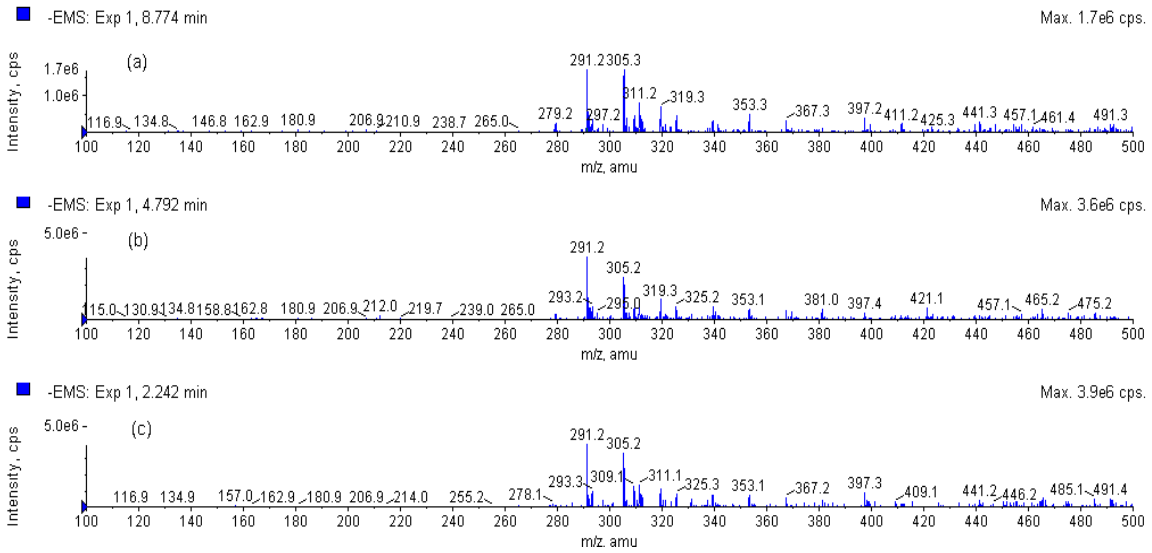


Figure 50: **EMS spectra of the other peaks found in Figure 38 at retention times (a) 8.7, (b) 4.7 and (c) 2.2 min. The same MS spectrum was observed indicating that no further PTPs could be detected**

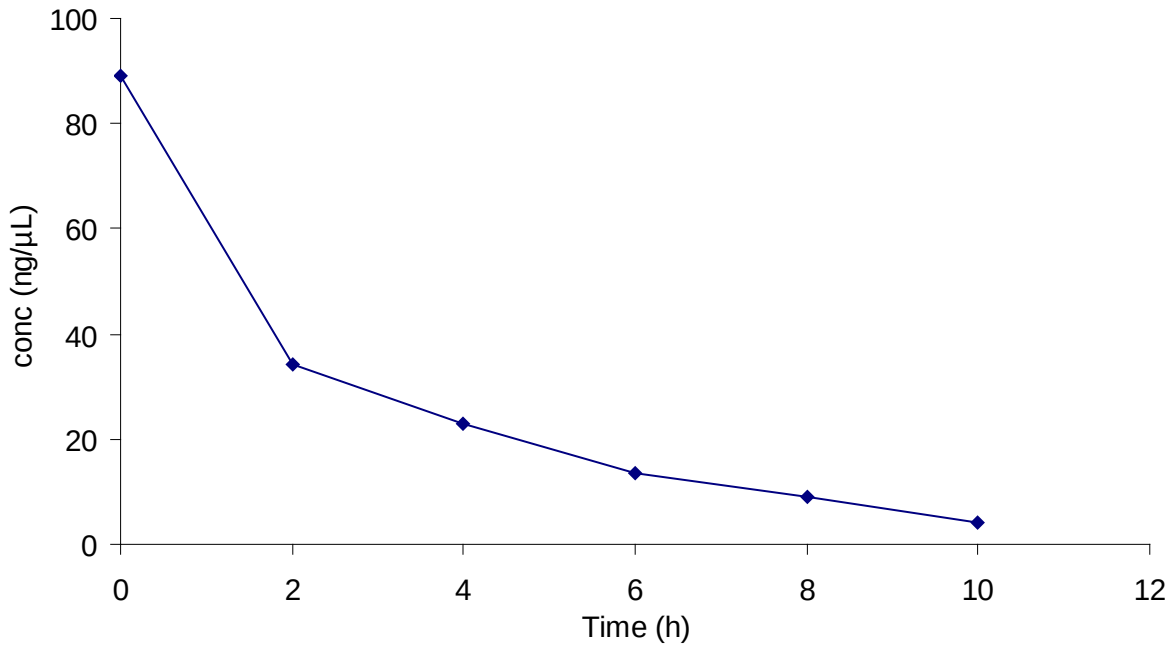
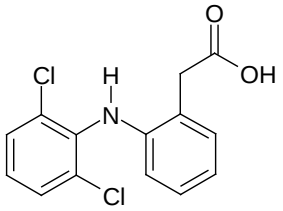
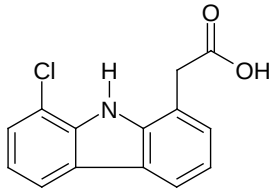
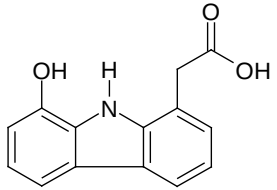
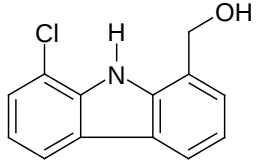


Figure 51: **Dissipation of diclofenac after irradiation in pure water for different intervals as detected by LC/MS/MS using the transition (m/z 294/250) in MRM mode**

Table 14: Diclofenac and its expected phototransformation products detected by LC/MS/MS

Structure	CAS	Molecular formula	Molecular mass	Precursor ion	Product ion	Retention time (min)
 2-[(2,6-Dichlorophenyl)amino] benzene acetic acid	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	295	294	250	15.3
 8-Chloro-9H-carbazole-1-acetic acid	131023-44-4	$C_{14}H_{10}ClNO_2$	259	258	214	11.1
 8-Hydroxy-9H-carbazole-1-acetic acid	131023-45-5	$C_{14}H_{11}NO_3$	241	240	196	5.2
 8-Chloro-9H-carbazole-1-methanol	212850-69-6	$C_{13}H_{10}ClNO$	231	229	185	3.1

4.8.3 Identification of transformation products of diclofenac in water/sediment systems

For identification of phototransformation products, diclofenac was irradiated in the water/sediment systems WS3 for 5 h. In contrast to the irradiation tests in pure water, here, only PTP1 could be detected besides diclofenac either in the water phases **[Figure 52]** or in the sediment extracts while PTP2 and 3 could not be detected. In the sediment extracts, however, diclofenac was found at much lower amounts (2 ± 0.2 %) than in the water phases (39 ± 2 %). The complete data set are listed in **Appendix, Table A32**. These results may reveal to PTP2 and 3 as the main reason for the formation of the non-extractable residues confirming the important role of sediment in the ecosystem. Therefore, sediments should not be disregarded when fate and behavior of polar compounds are to be monitored in aquatic compartments.

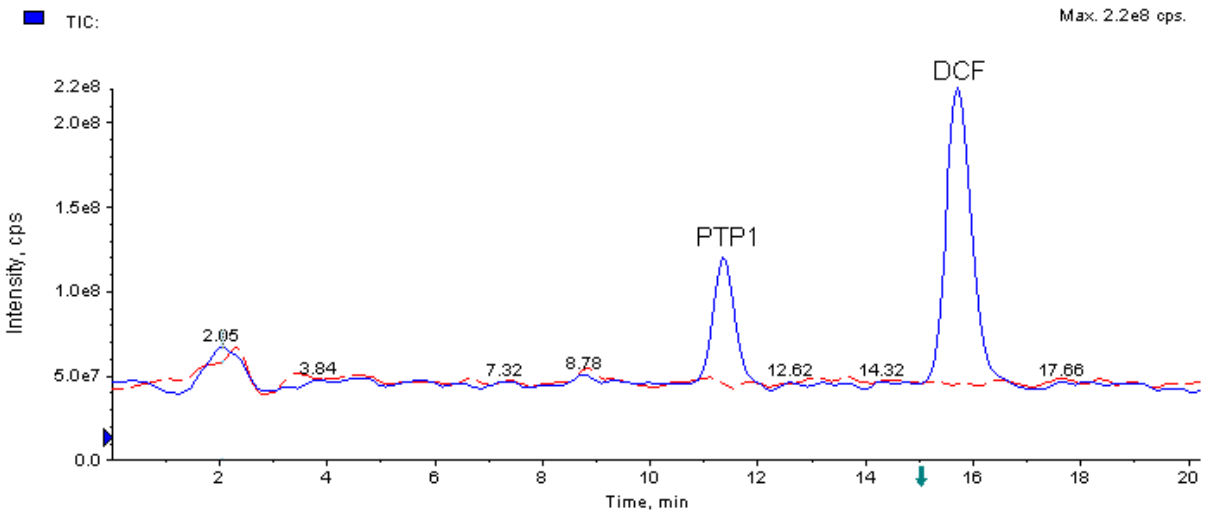


Figure 52: Smoothed total ion current chromatogram of blank (---), diclofenac (DCF) and 8-chloro-9H-carbazole-1-acetic acid (PTP1) (—) detected by LC/MS/MS using information depending acquisition method in the water phase of water/sediment WS3 after a 5 h irradiation period

On the other hand, water/sediment systems incubated for 7 days in the dark showed no indication for formation of biotransformation products under the experimental conditions. The reason might be attributed to the concentration of diclofenac which used in this experiment at higher level (3.3 mg/L) than in case of the radiotracer experiments (70 µg/L) where several unknowns were found to be formed as shown by RTLC. The transformation rate of diclofenac in sediment under aerobic condition was reported by Groning et al. (2007) to be inversely proportional to the concentration levels indicating a toxic effect of this drug or its resulting metabolites. The residual amounts of diclofenac were 59 ± 2 % and 19 ± 0.4 % for water

phase and sediment extracts, respectively, **[Appendix, Table A33]**. Losses of diclofenac within those tests may be also attributed to sorption processes in the sediment.

4.9 Structure confirmation of 8-chloro-9H-carbazole-1-acetic acid by 2D ^{1}H and ^{13}C NMR

In contrast to LC/MS/MS, NMR has higher limits of detection and analyses are to be carried out for single compounds or less complex mixtures. Therefore, an irradiation experiment at relatively high concentration of diclofenac was performed. As shown by reversed-phase thin layer chromatography and analytical HPLC/UVD, it was possible to enrich 8-chloro-9H-carbazole-1-acetic acid approximately to the 30-mg range to meet NMR's limits of detection. However, at such high concentration, diclofenac could not be completely separated and NMR analysis was performed for analytical solution containing diclofenac and 8-chloro-9H-carbazole-1-acetic acid. In contrast, at low concentration level of diclofenac, 8-chloro-9H-carbazole-1-acetic acid was completely separated (almost 100 % purity) as shown by HPLC/DAD **[Figure 53]**. Nevertheless, both compounds could be differentiated because the ^{1}H-NMR spectrum of 8-chloro-9H-carbazole-1-acetic acid differs from that of diclofenac mainly in two respects. First, the symmetrical aromatic three-spin system A2X spin system of the dichloroaniline moiety has turned into an unsymmetrical AMX spin system. Second, the four-spin system of the aminophenylacetic acid part has changed into a three-spin system **[Ernst, 2009]**. The chemical shifts of two most deshielded protons at δ = 7.95 and 7.98 ppm are compatible with those of H4 and H5 of a carbazole unit **[Figure 54]**. Structure of 8-chloro-9H-carbazole-1-acetic acid was thus proved by analysis of the ^{1}HNMR spectrum and by inspection of two-dimensional ^{1}H, ^{13}C hsqc and hmbc spectra **[Table 15]**.

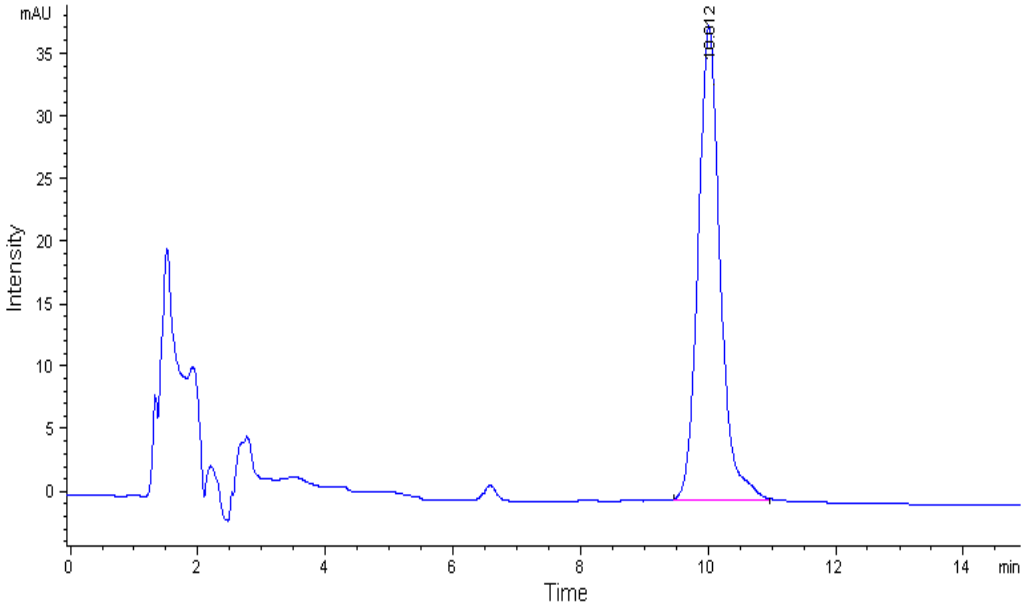


Figure 53: **UV chromatogram of PTP1 after isolation as detected by HPLC/DAD at λ = 242 nm using acetonitrile/water/formic acid (50: 50: 0.1, v/v/v) as mobile phase**

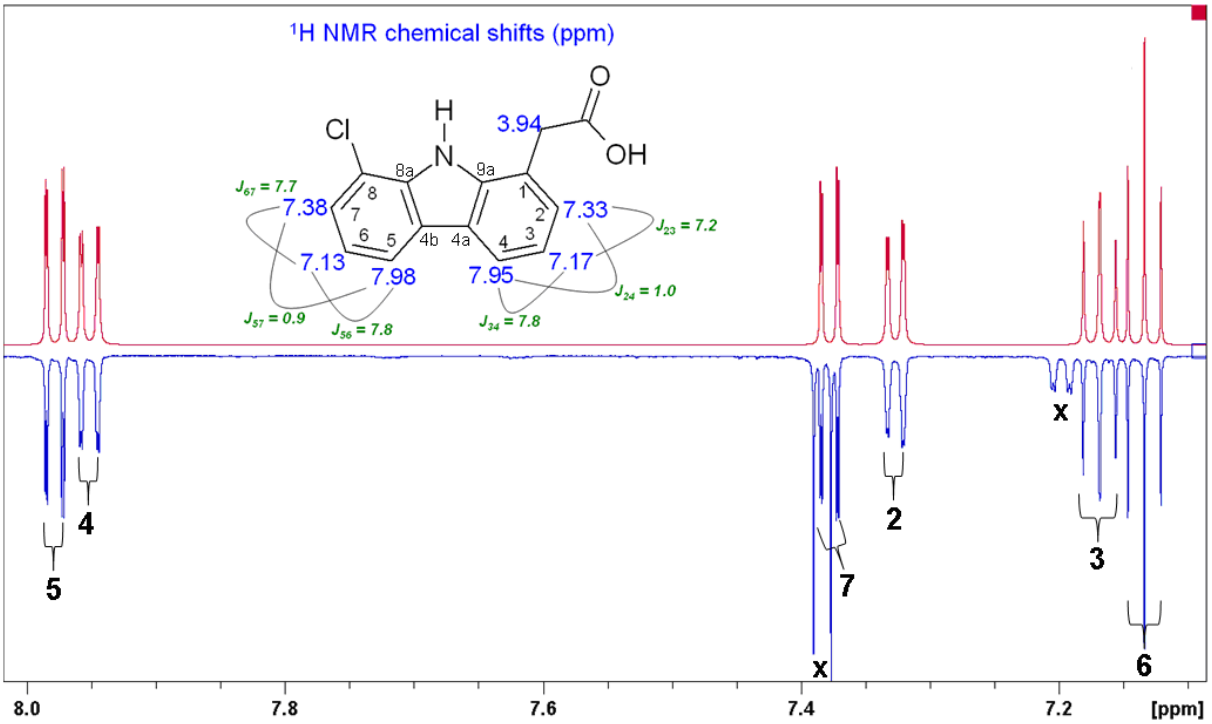


Figure 54: **Experimental (bottom) and simulated (top) 600 MHz ^{1}H-NMR spectra of PTP1 (aromatic region), solvent: CD_3OD. Signals marked with x are due to diclofenac**

Table 15: ^{1}H- and ^{13}C-NMR data of 8-chloro-9H-carbazole-1-acetic acid (PTP1), solvent: CD_3OD

Position	δ_H [a]	δ_C	hmbc[b] of ^{13}C with
1	---	121.2	CH_2
2	7.33	128.6	H4, CH_2
3	7.17	120.9	---
4	7.95	119.9	H2
4a	---	124.8	H3, H5
4b	---	126.6	H4, H6
5	7.98	119.7	H7
6	7.13	120.8	---
7	7.38	125.9	H5
8	---	117.3	H6
8a	---	138.5	H5, H7
9a	---	140.8	H2, H4, CH_2
CH_2	3.94	41.1	H2
COO		178.7	CH_2

[a] Coupling constants: J_{23} = 7.2, J_{24} = 1.0, J_{34} = 7.8, J_{56} = 7.8, J_{57} = 0.9, J_{67} = 7.7 Hz

[b] Only strongest crosspeaks given

5. Conclusions

Presence of pharmaceuticals in the environment is considered one of the hot topics recently acquired attention of many ecologists in different countries. Among the environmental issues, fate and behavior studies of several pharmaceuticals in water/sediment systems are poorly understood due to the lack of information. Pharmaceuticals could be potentially of concern in the aquatic environment due to their pharmacological actions and/or probably their transformation to perhaps more ecotoxic compounds. Therefore, fate studies are essential for estimation of various aspects such as persistence, occurrence, accumulation and estimation of the environmental risk assessment of pharmaceuticals.

In addition to application of radiotracer techniques, investigation of pharmaceuticals under different environmental conditions is very helpful in order to obtain comprehensive information and hence to find suitable methods for reducing their environmental risks. Mainly two conditions are essential to be tested when the fate of pharmaceuticals are to be investigated in the water/sediment system. First is the fate of pharmaceuticals in the upper layers of the water bodies and shallows which are mainly exposed to the natural sunlight. This can be simulated by laboratory irradiation experiments using simulated sunlight. Second is the fate of pharmaceuticals in the deep water layers and river bed which are mainly exposed to the sediment biomass. This can be simulated by dark experiments. Although fate study under anaerobic conditions is important, aerobic condition is more important in case of water/sediment systems simulating the real situation especially at the water sediment interface which represents the most relevance place for biotransformation.

Additionally, the relevance of the fate study is extremely increased when accompanied by other studies such as sorption/desorption experiments, identification and isolation of the unknown transformation products. Such combined studies are very helpful for better understanding of the results as well as for convincing data interpretations. Practically, several studies in different directions for many pharmaceuticals need a long time up to years and perhaps decades. In case of limited time, therefore, investigation of one pharmaceutical compound in depth is more realistic than to investigate several pharmaceutical compounds superficially leaving many open questions behind.

Data regarding fate of some pharmaceuticals in water/sediment system are available but not including diclofenac. Therefore, the investigation of diclofenac in water/sediment systems was necessary to estimate several aspects:

1- The fate of ^{14}C-diclofenac in aquatic ecosystems can be partly simulated by the standard water/sediment test.
2- So far biotransformation is also accompanied by phototransformation, advanced laboratory test systems have to be applied to complete environmental risk assessment of photosensitive substances.
3- Even in the sediments with low organic matter contents, sorption and remobilization of diclofenac in water/sediment systems are occurred to a moderate extent.
4- The sorption of diclofenac is a concentration dependent process and best fitted to the Freundlich isotherm model.
5- Water immiscible solvents could be the best solvents for determination of the ER during fate studies in water/sediment systems.
6- The fate of diclofenac in water/sediment system is greatly influenced by the environmental conditions especially presence and absence of light.
7- In the dark water/sediment system, biotransformation and to moderate extend sorption were found to be the concentration determining process for diclofenac.
8- Complete transformation of diclofenac to carbon dioxide and water occurs at relatively low amounts.
9- The concentration is the rate determining step of the biotransformation process of diclofenac while sediment characteristics, texture, total microbial activity, and type of incubation system were found to have no considerable impact.
10- The same persistence character for diclofenac could not be obtained by application of different persistence parameters.
11- In presence of light, the most significant phototransformation process of diclofenac is the photocyclization to the corresponding monochlorinated carbazole and monohydroxy carbazole.
12- The phototransformation process, which is mainly controlled by the irradiation time, is the concentration determining process for diclofenac in water/sediment systems as well as in pure and native water.
13- The transformation products of diclofenac display a higher potential for accumulation in the sediments compartments and the accumulated amounts are mainly controlled by the characteristic of the used sediments.
14- Sediments play an important role as a sink and at the same time as a source for polar transformation products of diclofenac.
15- Presence of sediments diversely affects the occurrence of the phototransformation products of diclofenac and hence should be involved in environmental monitoring programs.

16- Application of different analytical techniques is necessary for identification of transformation products, i.e., GC/MS, HPLC/UVD, HPLC/DAD, LC/MS/MS, and NMR.

17- In case of availability of alternative techniques, GC/MS should not be used in the analysis of diclofenac.

18- LC/MS/MS-QTRAP is a powerful technique for identification of unknowns as well as quantitation of known compounds.

19- The strategy used in the present study for identification of phototransformation of diclofenac which based on application of different scan modes can easily be applied for identification of any other unknown compound.

20- Further efforts should be done to make the transformation products available as authentic reference standards. Otherwise, method development for quantitative analysis would not be possible.

6. Summary and future perspectives

Recently, pharmaceuticals have been identified as a new class of environmental contaminants. Polarity, excessive use and insufficient treatment of either pharmaceutical industrial wastes or wastewater could be the main reasons for presence of considerable amounts of pharmaceutical residues in the aquatic environment. The non-steroidal anti-inflammatory drug diclofenac is considered one of the most environmentally relevant pharmaceutical compounds. It has been frequently detected in almost all environmental surveys at concentrations ranging from 0.001 µg/L to 30 µg/L.

Due to its importance, diclofenac was subjected in the present study to comprehensive investigations in water/sediment systems aiming for more accurate environmental risk assessment. Therefore, the following studies were included in the current work:

1- Biotransformation and photoinduced transformation tests accompanied by sorption/desorption experiments using radiotracer technique in water/sediment systems.
2- Identification of its phototransformation products in water and water/sediment systems using GC/MS, HPLC, and LC/MS/MS techniques.
3- Isolation and structural confirmation of the most abundant phototransformation product of diclofenac using preparative reversed-phase column chromatography and semi-preparative HPLC followed by ^{1}H- and ^{13}C-NMR analysis using 1D and 2D NMR techniques.

For the environmental risk assessment, fate of diclofenac in water/sediment systems is of major relevance. In accordance to regulatory recommendations, therefore, the OECD 308 water/sediment simulation test was performed under aerobic conditions. Within the 100-day incubation period, ^{14}C-diclofenac continuously disappeared from the aqueous phase while the residues increased in the sediment. After a first increase up to 22 %, the ethyl acetate extractable residues dropped to 0.2 % while the non-extractable residues continuously increased and finally reached 82 %. Mineralization increased up to 13 %. At least 28 days were needed for ^{14}C-diclofenac in order to almost disappear from the water phase. At maximum, the DT_{50} and DT_{90} of diclofenac were estimated at 13 and 42 days, respectively. In the worst case, these results indicated that diclofenac could be classified as moderately persistent pharmaceutical compound in dark regions of the aquatic environment.

Furthermore, laboratory batch experiments were conducted to study the sorption and desorption behavior of ^{14}C-diclofenac in addition to their isotherms. Distribution coefficient (K_d) of sorption onto the sediment was 5.5 L/kg. On the other hand, desorption percentage of ^{14}C-diclofenac from the sediment was found to be 20 %. The isotherms fitted by Freundlich equation showed that they were closed to linear. The Freundlich coefficient K_F values of adsorption and desorption were 7.5 and 15.8 $\mu g^{1-1/n}$ $L^{1/n}$/kg, respectively.

Since diclofenac is known as a photosensitive substance and may thus undergo phototransformation in surface water, water/sediment tests have been also conducted in a special irradiation apparatus to advance the fate monitoring under laboratory conditions. In contrast to the incubation in the dark, up to 70 % of non-extractable residues were formed under irradiation within 3 days even though the formation of more polar phototransformation products with lower affinity to the sediment was expected. Therefore, the identification of phototransformation products of diclofenac was demanded.

Because transformation processes may result in formation of unknown compounds with different physico-chemical properties, several techniques based on GC/MS, HPLC/UV, HPLC/DAD and LC/MS/MS were applied after numerous irradiation experiments either in pure water or in water/sediment systems for identifying phototransformation products of diclofenac. The analyses were carried out after enrichment of the analytes from the water phases and the sediments by means of liquid/liquid, solid phase or liquid/solid extraction.

In the irradiated water samples, phototransformation products could not be detected by means of GC/MS, even though polar analytes were derivatized by pentafluorobenzylation. This analysis was interfered because diclofenac underwent an intramolecular cyclization forming the thermal artifact 1-(2,6-dichlorophenyl)indolin-2-one in the split/splitless injector. In contrast, three UV detectable transformation products could be detected besides diclofenac when HPLC/UVD and HPLC/DAD were applied.

The structure elucidation of these compounds was then performed by LC/MS/MS analysis using for the first time a hybrid triple quadrupole linear ion trap mass spectrometer operated in negative electrospray ionization mode. Application of different scan techniques such as enhanced MS and enhanced product ion scan using information depending acquisition method followed by precursor ion scan were necessary for determination of the target nominal masses of the expected transformation compounds.

By means of mass spectra interpretation, the formation of 8-chloro-9H-carbazole-1-acetic acid, 8-hydroxy-9H-carbazole-1-acetic acid and 8-chloro-9H-carbazole-1-methanol was indicated in case of irradiated water samples. In contrast, only 8-chloro-9H-carbazole-1-acetic acid was found besides diclofenac in case of water/sediment irradiation experiments. These results indicated the impact of the sediment on fate and behavior of phototransformation products in aquatic environment and demonstrated that the formation of non-extractable residues in the sediment could be mainly attributed to 8-hydroxy-9H-carbazole-1-acetic acid and 8-chloro-9H-carbazole-1-methanol.

Due to the lack of authentic reference standards, isolation of phototransformation products was necessary for structural confirmation by means of NMR techniques. Because of its environmental relevance, the most abundant phototransformation product of diclofenac 8-chloro-9H-carbazole-1-acetic acid was primary enriched by means of preparative reversed phase column chromatography and semi-preparative HPLC. Finally, the structure was confirmed by NMR spectra interpretations where the ^{1}H and ^{13}C chemical shifts and the detected spin systems were found to be compatible with the proposed carbazole structure.

Data based on transformation of pharmaceuticals in water/sediment systems should be involved in the risk assessment procedure of diclofenac. For this purpose, further data on the fate in anaerobic sediments should be gathered. Biotransformation products of diclofenac in water/sediment systems should be structurally identified and prepared as authentic reference standards. Thereafter, thorough ecotoxicological tests have to be performed. Similarly, monitoring programs are strongly recommended for other pharmaceuticals detected as environmental contaminants. The results should be then used to list the pharmaceuticals priority to which the environmental risk assessment is necessary. Based on the risk quotient later on, risk reduction measures should be proposed, discussed, tested and finally applied.

7. References

Abdel-Hamid, M. E., Novotny, L., Hamza, H., 2001. Determination of diclofenac sodium, flufenamic acid, indomethacin and ketoprofen by LC-APCI-MS. Pharm. Biomed. Anal. 24, 587-594.

Adler, N. E., Koschorreck, J., Rechenberg, B., 2008. Environmental impact assessment and control of pharmaceuticals: The role of environmental agencies. Water Sci. Technol. 57, 91-97.

Agueera, A., Pérez Estrada, L. A., Ferrer, I., Thurman, E. M., Malato, S., Fernández-Alba, A. R., 2005. Application of time-of-flight mass spectrometry to the analysis of phototransformation products of diclofenac in water under natural sunlight. Mass Spec. 40, 908-915.

Ahrer, W., Scherwenk, E., Buchberger, W., 2001. Determination of drug residues in water by the combination of liquid chromatography or capillary electrophoresis with electrospray mass spectrometry. J. Chromatogr. A 910, 69-78.

Alcock, R. E., Sweetman, A., Jones, K. C., 1999. Assessment of organic contaminant fate in wastewater treatment plants. I: Selected compounds and physiochemical properties. Chemosphere 38, 2247-2262.

Al-Rifai, J. H., Gabelish, C. L., Schaefer, A. I., 2007. Occurrence of pharmaceutically active and non-steroidal estrogenic compounds in three different wastewater recycling schemes in Australia. Chemosphere 69, 803-815.

Andreozzi, R., Marotta, R., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Ankley, G. T., Brooks, B. W., Huggett, D. B., Sumpter, J. P., 2007. Repeating history: Pharmaceuticals in the environment. Environ. Sci. Technol. 41, 8211-8217.

Anon, 2005. Drugs in the environment - analysis of surface water and underground water by using LC-MS/MS method. Huanjing Huaxue 24, 491-493.

Antonic, J., Heath, E., 2007. Determination of NSAIDS in river sediment samples. Anal. Bioanal. Chem. 387, 1337-1342.

Arun, P. R., Azeez, P. A., 2004. Vulture population decline, diclofenac and avian gout. Curr. Sci. 87, 565-568.

Ashton, D., Hilton, M., Thomas, K., 2004. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. Sci. Total Environ. 333, 167-184.

Barron, L., Havel, J., Purcell, M., Szpak M., Kelleher, B., Paull, B., 2009. Predicting sorption of pharmaceuticals and personal care products onto soil and digested sludge using artificial neural networks. Analyst 134, 663-670.

Bartels, P., von Tuempling Jr., W., 2007. Solar radiation influence on the decomposition process of diclofenac in surface waters. Sci. Total Environ. 374, 143-155.

Batarseh, M. I., 2003. Residue analysis of organic pollutants in sediments from the Amman/Zarqa Area in Jordan. PhD Thesis, TU Braunschweig.

Belfroid, A. C., Van der Horst, A., Vethaak, A. D., Schaefer, A. J., Rijs, G. B. J., Cofino, W. P. 1999. Analysis and occurence of estrogenic hormones and their glucuronides in surface water and wastewater in The Netherlands. Sci. Total Environ. 225, 101-108.

Bendz, D., Paxeus, N. A., Ginn, T. R., Loge, F. J., 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Hoje River in Sweden. Hazard. Mat. 122, 195-204.

Benotti, M. J., Stanford, B. D., Wert, E. C., Snyder, S. A., 2009. Evaluation of a photocatalytic reactor membrane pilot system for the removal of pharmaceuticals and endocrine disrupting compounds from water. Water Res. 43, 1513-1522.

Bernhard, M., Mueller, J., Knepper, T. P., 2006. Biodegradation of persistent polar pollutants in wastewater: Comparison of an optimised lab-scale membrane bioreactor and activated sludge treatment. Water Res. 40, 3419-3428.

BLAC, 2003. Arzneimittel in der Umwelt. Auswertung der Untersuchungsergebnisse. http://www.blak-uis.de/servlet/is/2146/P-2c.pdf.

Bort, R., Ponsoda, X., Jover, R., Gomez-Lechon, J., Castell, J. V., 1998. Diclofenac toxicity to hepatocytes: A role for drug metabolism in cell toxicity. Pharm. Exp. Ther. 288, 65-72.

Bowen, E. J., Eland, J. H. D., 1963. Photochemistry of diphenylamine solutions. Proc. Chem. Soc (London). 202.

Boyd, G. R., Reemtsma, H., Grimm, D. A., Mitra, S., 2003. Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA, and Ontario, Canada. Sci. Total Environ. 311, 135-149.

Braga, O., Smythe, G. A., Schafer, A., Feitz, A. J., 2005. Fate of steroid estrogens in Australian inland and coastal wastewater treatment plants. Environ. Sci. Technol. 39, 3351-3358.

Brain, R. A., Johnson, D. J., Richards, S. M., Sanderson, H., Sibley, P. K., Solomon, K. R., 2004. Effects of 25 pharmaceutical compounds to *Lemna gibba* using a seven-day static-renewal test. Environ. Toxicol. Chem. 23, 371-382.

Brian, J. V., Harris, C. A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfa, A., Marcomini, A., Sumpter, J. P., 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ. Health Perspect. 113, 721-728.

Brooks, B. W., Chambliss, C. K., Stanley, J. K., Ramirez, A., Banks, K., Johnson, R., 2005. Determination of selected antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24, 464-469.

Bueno, M. J. M., Aguera, A., Gomez, M. J., Hernando, M. D., Garcia-Reyes, J. F., Fernandez-Alba, A. R., 2007. Application of liquid chromatography/quadrupole-linear ion trap mass spectrometry and time-of-flight mass spectrometry to the determination of pharmaceuticals and related contaminants in wastewater. Anal. Chem. 79, 9372-9384.

Bui, T. X., Choi, H., 2009. Adsorptive removal of selected pharmaceuticals by mesoporous silica SBA-15. Hazard. Mat. 168, 602-608.

Buser, H. R., Mueller, M. D., Theobald, N., 1998a. Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. Environ. Sci. Technol. 32, 188-192.

Buser, H. R., Poiger, T., Mueller, M. D., 1998b. Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: Rapid photodegradation in a lake. Environ. Sci. Technol. 32, 3449-3456.

Campagnolo, E. R., Johnson, K. R., Karpati, A., Rubin, C. S., Kolpin, D. W., Meyer, M. T., Esteban, J. E., Currier, R. W., Smith, K., Thu, K. M., McGeehin, M., 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. Sci. Total Environ. 299, 89-95.

Carballa, M., Fink, G., Omil, F., Lema, J. M., Ternes, T., 2008. Determination of the solid-water distribution coefficient (Kd) for pharmaceuticals, estrogens and musk fragrances in digested sludge. Water Res. 42, 287-295.

Carballa, M., Omil, F., Lema, J. M., 2005. Removal of cosmetic ingredients and pharmaceuticals in sewage primary treatment. Water Res. 39, 4790-4796.

Carballa, M., Omil, F., Ternes, T., Lema, J. M., 2007. Fate of pharmaceutical and personal care products (PPCPs) during anaerobic digestion of sewage sludge. Water Res. 41, 2139-2150.

Carlsson, C., Johansson, A. K., Alvan, G., Bergman, K., Kuehler, T., 2006a. Are pharmaceuticals potent environmental pollutants? Part I: Environmental risk assessments of selected active pharmaceutical ingredients. Sci. Total Environ. 364, 67-87.

Carlsson, C., Johansson, A. K., Alvan, G., Bergman, K., Kuehler, T., 2006b. Are pharmaceuticals potent environmental pollutants? Part II: Environmental risk assessments of selected pharmaceutical excipients. Sci. Total Environ. 364, 88-95.

Castiglioni, S., Fanelli, R., Calamari, D., Bagnati, R., Zuccato, E., 2004. Methodological approaches for studying pharmaceuticals in the environment by comparing predicted and measured concentrations in River Po, Italy. Regul. Toxicol. Pharmacol. 39, 25-32.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography–tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. J. Chromatogr. A 1092, 206-215.

Castiglioni, S., Bagnati, R., Fanelli, R., Pomati, F., Calamari, D., Zuccato, E., 2006. Removal of pharmaceuticals in sewage treatment plants in Italy. Environ. Sci. Technol. 40, 357-363.

Cavalheiro, A. A., Bruno,J. C., Saeki, M. J., Valente, J. P. S., Florentino, A. O., 2008. Photocatalytic decomposition of diclofenac potassium usingsilver-modified TiO_2 thin films. Thin Solid Films 516, 6240-6244.

Chefetz, B., Mualem T., Ben-Ari J., 2008. Sorption and mobility of pharmaceutical compounds in soil irrigated with reclaimed wastewater. Chemosphere 73, 1335-1343.

ChemIDPlus, http://chem.sis.nlm.nih.gov/chemidplus/. Accessed 2010.

Chen, H. C., Wang, P. L., Ding, W. H., 2008. Using liquid chromatography–ion trap mass spectrometry to determine pharmaceutical residues in Taiwanese rivers and wastewaters. Chemosphere 72, 863-869.

Clara, M., Strenn, B., Kreuzinger, N., 2004. Carbamazepine as a possible anthropogenic marker in the aquatic environment: Investigations on the behavior of carbamazepine in wastewater treatment and during groundwater infiltration. Water Res. 38, 947-954.

Clara, M., Kreuzinger, N., Strenn, B., Gans, O., Kroiss, H., 2005a. The solids retention time – a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. Water Res. 39, 97-106.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., Kroiss, H., 2005b. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Res. 39, 4797-4807.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185-194.

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. 59, 309-315.

Comoretto, L., Chiron, S., 2005. Comparing pharmaceutical and pesticide loads into a small Mediterranean river. Sci. Total Environ. 349, 201-210.

Craven, D. B., Jahnke, R. A., Carlucci, A. F., 1986. Fine-scale distribution of microbial biomass and activity in California Borderland sediments. Deep Sea Res. 33, 379-390.

Cuthbert, R., Green, D. R., Ranada, S., Saravanan, S., Pain, D., Prakash, V., Cunningham, A. A., 2006. Rapid population declines of Egyptian Vulture (*Neophron Percnopterus*) and Red-Headed Vulture (*Sarcogyps Calvus*) in India. Anim. Conserv. 9, 349-354.

De Wever, H., Weiss, S., Reemtsma, T., Vereecken, J., Mueller, J., Knepper, T., Roerden, O., Gonzalez, S., Barcelo, D., Hernando, M. D., 2007. Comparison of sulfonated and other micropollutants removal in membrane bioreactor and conventional wastewater treatment. Water Res. 41, 935-945.

Deng, A., Himmelsbach, M., Zhu, Q., Frey, S., Sengl, M., Buchberger, W., Niessner, R., Knopp, D., 2003. Residue analysis of the pharmaceutical diclofenac in different water types using ELISA and GC-MS. Environ. Sci. Technol. 37, 3422-3429.

Drewes, J. E., Heberer, T., Rauch, T., Reddersen, K., 2003. Fate of pharmaceuticals during ground water recharge. Ground Water Monit. Rem. 23, 64-72.

Drillia, P., Stamatelatou, K., Lyberatos, G., 2003. Mobility of pharmaceuticals in soil. Proceedings of the International Conference on Environmental Science and Technology, 8th, Myrina, Lemnos Island, Greece, Sept. 8-10, Meeting Date 2003.

Drover, V. J., Bottaro, C. S., 2008. Determination of pharmaceuticals in drinking water by CD-modified MEKC: Separation optimization using experimental design. Sep. Sci. 31, 3740-3748.

Duong, H. A., Pham, N. H., Nguyen, H. T., Hoang, T. T., Pham, H. V., Pham, V. C., Berg, M., Giger, W., Alder, A. C., 2008. Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. Chemosphere 72, 968-973.

El Haj, B. M., Ainri, A. M. A., Hassan, M. H., Khadem, R. K. B., Marzouq, M. S., 1999. The GC/MS analysis of some commonly used non-steroidal anti-inflammatory drugs (NSAIDs) in pharmaceutical dosage forms and in urine. Forensic Sci. Int. 105, 141-153.

Elbaz-Poulichet, F., Seidel, J., Othoniel, C., 2002. Occurrence of an anthropogenic gadolinium anomaly in river and coastal waters of Southern France. Water Res. 36, 1102-1105.

EMEA, 2001. European Agency for the Evaluation of Medicinal Products. Discussion paper on environmental risk assessment of medicinal products for human use. C2/JCD/csteeop/ CPMPpaperRAssess-HumPharm12062001/D(01).

EMEA, 2006. European Agency for the Evaluation of Medicinal Products. Guideline on the environmental risk assessment for medicinal products for human use; CHMP/SWP/4447/00; www.emea.eu.int/pdfs/human/swp/444700en. pdf.

Encinas, S., Bosca, F., Miranda, M. A., 1998a. Photochemistry of 2,6-dichlorodiphenylamine and 1-chlorocarbazole, the photoactive chromophores of diclofenac, meclofenamic acid and their major photoproducts. Photochem. Photobiol. 68, 640-645.

Encinas, S., Bosca, F., Miranda, M. A., 1998b. Phototoxicity associated with diclofenac: Aphotophysical, photochemical, and photobiological study on the drug and its photoproducts. Chem. Res. Toxicol.11, 946-952.

Ericson, J. F., 2007. An evaluation of the OECD 308 water/sediment systems for investigating the biodegradation of pharmaceuticals. Environ. Sci. Technol., 41, 5803-5811.

Ernst, L, 2009. NMR laboratories of the Chemical Institutes, TU Braunschweig. Personal communication.

European Commission, 2006. Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of 18 December 2006 Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Establishing a European Chemicals Agency, Amending Directive 1999/45/EC and Repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.

European Pharmacopoeia, 2005. Council of Europe (COE) – European directorate for the quality of medicines and healthcare (EDQM), fifth ed. 1532.

Farre, M. l., Ferrer, I., Ginebreda, A., Figueras, M., Olivella, L., Tirapu, L., Vilanova, M., Barcelo, D., 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. J. Chromatogr. A 938, 187-197.

Fatta, D., Nikolaou, A., Achilleos, A., Meric, S., 2007. Analytical methods for tracing pharmaceutical residues in water and wastewater. Trends Anal. Chem. 26, 515–533.

Federal Biological Research Center for Agriculture and Forestry, 1998. Data Requirements and Criteria for Decision-making in the European Union and the Federal Republic of Germany for the Authorization Procedure of Plant protection products. Mitt. a. d. Biol. Bundesanst. 358, 63-68.

Felis, E., Miksch, K., 2009. Removal of analgesic drugs from the aquatic environment using photochemical methods. Water Sci. Technol. 60, 2253-2259.

Felis, E., Wiszniowski, J., Miksch, K., 2009. Advanced oxidation of diclofenac in various aquatic environments. Arch. Environ. Prot. 35, 15-26.

Fent, K., Weston, A. A., Caminada, D. 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122-159.

Ferrari, B., Paxeus, N., Lo Giudice, R., Pollio, A., Garric, J., 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: Study of carbamazepine, clofibric acid, and diclofenac. Ecotox. Environ. Saf. 55, 359-370.

Fichter, P. M., Holden, P. W., 1992. A field study to meet United States Environmental Protection Agency regulatory requirements for measurement of movement of pesticides to ground water. Brighton Crop Protect. Conf. Pests and Diseases. 2, 853-858.

Fraenzle, O., Kuhnt, G., Vetter, L., (1987). Selection of representative soils in the EC territory. UBA Report 106 02 045, Part I.

Galmier, M. J., Bouchon, B., Madelmont, J. C., Mercier, F., Pilotaz, F. C., Lartigue, C., 2005. Identification of degradation products of diclofenac by electrospray ion trap mass spectrometry. Pharm. Biomed. Anal. 38, 790-796.

Garcia-Araya, J. F., Beltran, F. J., Aguinaco, A., 2010. Diclofenac removal from water by ozone and photolytic TiO_2 catalysed processes. Chem. Technol. Biotechnol. 85, 798-804.

Garrison, A. W., Pope, J. D., Allen, F. R., 1976. GC/MS analysis of organic compounds in domestic wastewaters. In Identification and analysis of organic pollutants in water Keith, C. H., Ed., Ann Arbor Science Publishers: Ann Arbor, MI, USA. 517-556.

Gaudiano, M. C., Valvo, L., Bertocchi, P., Manna, L., 2003. RP-HPLC study of the degradation of diclofenac and piroxicam in the presence of hydroxyl radicals. Pharm. Biomed. 32, 151-158.

Gevao, B., Semple, K. T., Jones, K. C., 2000. Bound pesticide residues in soils: A review. Environ. Poll. 108, 3-14.

Giordano, F., Rossi, A., Pasquali, I., Bettini, R., Frigo, E., Gazzaniga, A., Sangalli, M. E., Mileo, V., Catinella, S., 2003. Thermal degradation and melting point determination of diclofenac. Therm. Anal. Cal. 73, 509-518.

Glaeske, G., Janhsen, K., GEK Arzneimittelreport 2006. Schriftenreihe zur Gesundheitsanalyse, Vol. 44. Asgard Verlag, Hippe (2006)

Golet, E. M., Alder, A. C., Hartmann, A., Ternes, T. A., Giger, W., 2001. Trace determination of fluoroquinolone antibacterial agents in urban wastewater by solid-phase extraction and liquid chromatography with fluorescence detection. Anal. Chem. 73, 3632-3638.

Golet, E. M, Alder, A. C, Giger W., 2002. Environmental exposure and risk assessment of fluoroquinolone antibacterial agents in wastewater and river water of the Glatt Valley Watershed, Switzerland. Environ. Sci. Technol. 36, 3645-3651.

Golet, E. M., Xifra, I., Siegrist, H., Alder, A. C., Giger, W., 2003. Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. Environ. Sci. Technol. 37, 3243-3249.

Gomez, M. J., Martinez Bueno, M. J., Lacorte, S., Fernández-Alba, A. R., Agueera, A., 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. Chemosphere 66, 993-1002.

Gomez, M. J., Petrovic, M., Fernandez-Alba, A. R., Barcel, D., 2006. Determination of pharmaceuticals of various therapeutic classes by solid phase extraction and liquid chromatography tandem mass spectrometry analysis in hospital effluent wastewaters. J. Chromtogr. A 1114, 224-233.

Goss, K. U., Schwarzenbach, R. P., 2001. Linear free energy relationships used to evaluate equilibrium partitioning of organic compounds. Environ. Sci. Technol. 35, 1-9.

Green, R. E., Newton, I., Shultz, S., Cunningham, A. A., Gilbert, M., Pain, D. J., Prakash, V., 2004. Diclofenac poisoning as a cause of vulture population declines across the Indian subcontinent. Appl. Ecol. 41, 793-800.

Green, R. E., Taggart, M. A., Das, D., Pain, D. J., Kumar, C. S., Cunningham, A. A., Cuthbert, R., 2006. Collapse of Asian vulture populations: Risk of mortality from residues of the veterinary drug diclofenac in carcasses of treated cattle. Appl. Ecol. 43, 949-956.

Groening, J., Held, C., Garten, C., Claussnitzer, U., Kaschabek, S. R., Schloemann, M., 2007. Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate. Chemosphere 69, 509-516.

Gros, M., Petrovic, M., Barcelo, D., 2006. Development of a multi-residue analytical methodology based on liquid chromatography tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. Talanta 70, 678-690.

Grung, M., Kaellqvist, T., Sakshaug, S., Skurtveit, S., Thomas, K. V., 2008. Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline. Ecotoxicol. Environ. Saf. 71, 328-340.

Haider, K., Spiteller, M., Reichert, K., Fild, M., 1992. Derivatization of humic compounds: An analytical approach for bound organic residues. Environ. Anal. Chem. 46, 201-211.

Hajare, A. A., Pishawikar, S. A., 2006. Comparative invitro adsorption studies of diclofenac sodium and diltiazem hydrochloride by talc. Ind. pharm. 81-84.

Hajkova, R., Solich, P., Pospisilova, M., Sicha, J., 2002. Simultaneous determination of methylparaben, propylparaben, sodium diclofenac and its degradation product in a topical emulgel by reversed-phase liquid chromatography. Anal. Chim. Acta. 467, 91-96.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Luetzhoft, H. C., Jorgensen, S. E., 1997. Occurrence, fate and effects of pharmaceutical substances in the environment - a review. Chemosphere 36, 357-393.

Hartig, C., Storm, T., Jekel, M., 1999. Detection and identification of sulphonamide drugs in municipal wastewater by liquid chromatography coupled with electrospray ionisation tandem mass spectrometry. J. Chromatogr. A 854, 163-173.

Hartmann, A., Alder, A. C, Koller, T., Widmer, R. M., 1998. Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater. Environ. Toxicol. Chem. 17, 77-382.

Hartmann, J., Bartels, P., Mau, U., Witter, M., von Tuempling, W., Hofmann, J., Nietzschmann, E., 2008. Degradation of the drug diclofenac in water by sonolysis in presence of catalysts. Chemosphere 70, 453-461.

Heberer, T., 1995. Identification and quantification of pesticide residues and environmental contaminants in ground and surface water applying capillary gas chromatography-mass spectrometry. Wissenschaft and Technik Verlag, Berlin, (1995) 437.

Heberer, T., Stan, H. J., 1997. Determination of clofibric acid and N-(phenylsulfonyl)-sarcosine in sewage, river and drinking water. Environ. Anal. Chem. 67, 113-124.

Heberer, T., Dunnbier, U., Reilich, C., Stan, H. J., 1997. Detection of drugs and drug metabolites in groundwater samples of a drinking water treatment plant. Fresenius Environ. Bull. 6, 6438-443.

Heberer, T., Schmidt-Baeumler, K., Stan, H. J., 1998. Occurrence and distribution of organic contaminants in the aquatic system in Berlin, Germany. Part 1: Drug residues and other polar contaminants in Berlin surface and groundwater. Acta Hydroch. Hydrobio. 26, 272-278.

Heberer, T., Verstraeten, I. M., Meyer, M. T., Mechlinski, A., Reddersen, K., 2001. Occurrence and fate of pharmaceuticals during bank filtration preliminary results from investigations in Germany and the United States. Water Resour. Update 120, 4-17.

Heberer, T., Reddersen, K., Mechlinski, A., 2002. From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas. Water Sci. Technol. 46, 81-88.

Heberer, T., 2002a. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: A review of recent research data. Toxicol. Lett. 131, 5-17.

Heberer, T., 2002b. Tracking persistent pharmaceutical residues from municipal sewage to drinking water. J. Hydrol. 266, 175-189.

Heberer, T., Mechlinski, A., Fanck, B., Knappe, A., 2004. Field studies on the fate and transport of pharmaceutical residues in bank filtration. Ground Water Monit. Rem. 24, 70-77.

Heberer, T., Feldmann, D., 2005. Contribution of effluents from hospitals and private households to the total loads of diclofenac and carbamazepine in municipal sewage effluents-modeling versus measurements. Hazard. Mat. 122, 211-218.

Heise, J., Hoeltge, S., Schrader, S., Kreuzig, R., 2006. Chemical and biological characterization of non-extractable sulfonamide residues in soil. Chemosphere 65, 2352-2357.

Hernando, M. D., Heath,. E., Petrovic, M., Barcelo, D., 2006. Trace level determination of pharmaceutical residues by LC-MS/MS in natural and treated waters. A pilot survey study. Anal. Bioanal. Chem. 385, 985-991.

Higaite, C., Azarnoff, D. L., 1977. Drugs and drug metabolites as environmental contaminants: Chlorophenoxyisobutyrate and salicylic acid in sewage water effluent. Life Sci. 20, 337-342.

Hilton, M. J., Thomas, K. V., 2003. Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography-electrospray tandem mass spectrometry. J. Chromatogr. A 1015, 129-141.

Hirsch, R, Ternes, T. A., Haberer, K., Mehlich, A., Ballwanz, F., Kratz, K. L., 1998. Determination of antibiotics in different water compartments via liquid chromatography electrospray tandem mass spectrometry. J. Chromatogr. A 815, 213-223.

Hirsch, R., Ternes, T. A., Haberer, K., Kratz, K. L., 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225, 109-118.

Hirsch, R., Ternes, T. A., Lindart, A., Haberer, K., Wilken, R. D., 2000. A sensitive method for the determination of iodine containing diagnostic agents in aqueous matrices using LC-electrospray-tandem-MS detection. Fresenius Anal. Chem. 366, 835-841.

Hoeger, B., Koellner, B., Dietrich, D.R., Hitzfeld, B., 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). Aquat. Toxicol. 75, 53-64.

Hoellrigl-Rosta, A., Kreuzig, R., Bahadir, M., 1999. Investigations on the metabolic fate of prochloraz in soil under field and laboratory conditions. Pestic. Sci. 55, 531-538.

Holm, J. V., Ruegge, K., Bjerg, P. L., Christensen, T. H., 1995. Occurrence and distribution of pharmaceutical organic compounds in the groundwater downgradient of a landfill (Grindsted, Denmark). Environ. Sci. Technol. 29, 1415-1420.

Hong, H. N., Kim, H. N., Park, K. S., Lee, S. K., Gu, M. B., 2007. Analysis of the effects diclofenac has on Japanese medaka (*Oryzias latipes*) using real-time PCR. Chemosphere 67, 2115-2121.

Huang, P. M., Wang, T. S. C., Wang, M. K., Wu, M. H., Hsu, N. W., 1977. Retention of phenolic acids by noncrystalline hydroxyaluminum and iron compounds and clay minerals of soils. Soil Sci. 123, 213-219.

Huber, M., Canonica, S., Park, G. Y., von Gunten, U., 2003. Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. Environ. Sci. Technol. 37, 1016-1024.

Huschek, G., Krengel, D., 2002. Mengenermittlung und Systematisierung von Arzneimittelwirkstoffen im Rahmen der Umweltprüfung von Human- und Tierarzneimittel gemaess 28 AMG. Forschungsbericht 20067401 Umweltbundesamt Dessau, Germany.

Huschek, G., Hansen, P. D., Maurer, H. H., Krengel, D., Kayser, A., 2004. Environmental risk assessment of medicinal products for human use according to European Commission recommendations. Environ. Toxicol. 19, 226-240.

Ibrahim, A. R. S., El-Feraly, F. S., Al-Said, M. S., 1996. Microbiological transformation of diclofenac. Saudi. Pharm. 4, 165-169.

Iqbal, M. J., Chaudhry, M. A., 2009. Thermodynamic study of three pharmacologically significant drugs: Density, viscosity, and refractive index measurements at different temperatures. Chem. Thermodyn. 41, 221-226.

ISO 14240-1 and 2, 1997. Soil Quality - Determination of soil microbial biomass. Part 1: Substrate-induced respiration method.

Jagger, J., 1985. Solar UV actions on living cells. New York: Praeger Scientific 1-10.

Johnson, A. C., Keller, V., Williams, R. J., Young, A. A., 2007. Practical demonstration in modelling diclofenac and propranolol river water concentrations using a GIS hydrology model in a rural UK catchment. Environ. Poll. 146, 155-165.

Jones, O. A. H., Voulvoulis, N., Lester, J. N., 2002. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Res. 36, 5013-5022.

Jones, O. A. H., Voulvoulis, N. and Lester, J. N., 2001. Human pharmaceuticals in the aquatic environment- a review. Environ. Technol. 22, 1383-1394.

Joss, A., Zabczynski, S., Goebel, A., Hoffmann, B., Loeffler, D., McArdell, C. S., Ternes, T. A., Thomsen, A., Siegrist, H., 2006. Biological degradation of pharmaceuticals in municipal wastewater treatment: proposing a classification scheme. Water Res. 40, 1686-1696.

Joss, A., Keller, E., Alder, A. C., Gobel, A., McArdell, C. S., Ternes, T., Siegrist, H., 2005. Removal of pharmaceuticals and fragrances in biological wastewater treatment. Water Res. 39, 3139-3152.

Jux, U., Baginski, M., Arnold, H. G., Kroenke, M., Seng, P. N., 2002. Detection of pharmaceutical contaminants of river, pond, and tap water from Cologne (Germany) and surroundings. Hyg. Environ. Health. 205, 393-398.

Khalaf, H., Salste, L., Karlsson, P., Ivarsson, P., Jass, J., Olsson, P. E., 2009. In vitro analysis of inflammatory responses following environmental exposure to pharmaceuticals and inland waters. Sci. Total Environ. 407, 1452-1460.

Khan, S. J., Ongerth, J. E., 2004. Modelling of pharmaceutical residues in Australian sewage by quantities of use and fugacity calculations. Chemosphere 54, 355-367.

Kim, I., Tanaka, H., 2009. Photodegradation characteristics of PPCPs in water with UV treatment. Environ. Int. 35, 793-802.

Kime, D. E., Nash, J. P., 1999. Gamete viability as an indicator of reproductive endocrine disruption in fish. Sci. Total. Environ. 233, 123-129.

Kimura, K., Hara, H., Watanabe, Y., 2007. Elimination of selected acidic pharmaceuticals from municipal wastewater by an activated sludge system and membrane bioreactors. Environ. Sci. Technol. 41, 3708-3714.

King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C., Olah, T., 2000. Mechanistic investigation of ionization suppression in electrospray ionization. Am. Soc. Mass Spectrom. 11, 942-950.

Klaus, U., Oesterreich, T., Volk, M., Spiteller, M., 1998. Interaction of aquatic dissolved organic matter (DOM) with amitrole: The nature of the bound residues. Acta Hydrochim. Hydrobiol. 26, 311-317.

Knopp, D., Deng, A., Letzel, M., Taggart, M., Himmelsbach, M., Zhu, Q. Z., 2007 Immunological determination of the pharmaceutical diclofenac in environmental and biological samples. Rational environmental management of agrochemicals: Risk assessment, monitoring and remedial action, ACS symposium series book 966, 203-326.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., Buxton, H. T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. Environ. Sci. Technol., 36, 1202-1211.

Kosjek, T., Health, E., Krbavcic, A., 2005. Determination of non-steroidal anti-inflammatory drug (NSAID) residus in water samples. Environ. Int. 31, 679-685.

Kosjek, T., Heath, E., Kompare, B., 2007a. Removal of pharmaceutical residues in a pilot wastewater treatment plant. Anal. Bioanal. Chem. 387, 1379-1387.

Kosjek, T., Heath, E., Petrovic, M., Barcelo, D., 2007b. Mass spectrometry for identifying pharmaceutical biotransformation products in the environment. Trends Anal. Chem. 26, 1076-1085.

Kosjek, T., Zigon, D., Kralj, B., Heath, E., 2008. The use of quadrupole-time-of-flight mass spectrometer for the elucidation of diclofenac biotransformation products in wastewater. J. Chromatogr. A 1215, 57-63.

Kosjek, T., Heath, E., Perez, S., Petrovic, M., Barcelo, D., 2009. Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole time-of-flight mass spectrometry. J. Hydrol. 372, 109-117.

Kot-Wasik, A., Debska, J., Wasik, A., Namiesnik, J., 2006. Determination of nonsteroidal anti-inflammatory drugs in natural waters using off-line and on-line SPE followed by LC coupled with DAD-MS. Chromatographia 64, 13-21.

Koutsouba, V., Heberer, T., Fuhrmann, B., Schmidt-Baumler, K., Tsipi, D., Hiskia, A., 2003. Determination of polar pharmaceuticals in sewage water of Greece by gas chromatography-mass spectrometry. Chemosphere 51, 69-75.

Kreuzig, R., 1998. Entwicklung analytischer Methoden zur Differenzierung von Abbau und Sorption als konzentrationsbestimmenden Prozessen für Pflanzenschutzmittel-Wirkstoffe in Boeden. Habilitationsschrift, TU Braunschweig; ISBN 3-89720-291-3, 1-189.

Kreuzig, R., Hoeltge, S., 2005. Investigations on the fate of sulfadiazine in manured soils: Laboratory experiments and test plot studies. Environ. Toxicol. Chem. 24, 260-265.

Kreuzig, R., Hoeltge, S., Heise, J., Schmanteck, I., Stein, F., Batarseh, M. (2007): Veterinary Medicinal Products in Manures and Manured Soils: Development of a Technical Protocol for Laboratory Tests. UBA-Texte 45/07, ISSN 1862-4804. Umweltbundesamt, Dessau-Roßlau, 1-142.

Kreuzig, R., Kullmer, C., Matthies, B., Hoeltge, S., Dieckmann, H., 2003. Fate and behavior of pharmaceutical residues in soils. Fresenius Environ. Bull. 12, 550-558.

Kuemmerer, K., 2001. Drugs in the environment: Emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. Chemosphere 45, 957-969.

Kuemmerer, K. (Ed.), 2004. Pharmaceuticals in the Environment: Sources, fate, effects and risks, second ed., Springer, Berlin, Heidelberg.

Kunkel, U., Radke, M., 2008. Biodegradation of acidic pharmaceuticals in bed sediments: Insight from a laboratory experiment. Environ. Sci. Technol. 42, 7273-7279.

Landsdorp, D., Vree, T. B., Janssen, T. J., Guelen, P.J., 1990. Pharmacokinetics of retal diclofenac and its hydroxy metabolites in man. Clin. Pharmacol. Ther. Toxicol. 28, 298-302.

Larsson, D. G. J., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. Hazard. Mat. 148, 751-755.

Lee, R. B., Sarafin, K., Peart, T. E., Svoboda, M. L., 2003. Acidic pharmaceuticals in sewages: Methodology, stability test, occurrence, and removal from Ontario samples. Water Qual. Res. J. Can. 38, 667-682.

Leemann, T., Transon, C., Dayer, P., 1993. Cytochrome P450TB(CYP2C): A major monooxygenase catalyzing diclofenac 40-hydroxylationin human liver. Life Sci. 52, 29-34.

Letzel, M., Metzner, G., Letzel, T. 2009. Exposure assessment of the pharmaceutical diclofenac based on long-term measurements of the aquatic input. Environ. Int. 35, 363-368.

Lin, A. Y. C., Yu, T. H., Lin, C. F., 2008. Pharmaceutical contamination in residential, industrial, and agricultural waste streams: Risk to aqueous environments in Taiwan. Chemosphere 74, 131-141.

Lindqvist, N., Tuhkanen, T., Kronberg, L., 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. Water Res. 39, 2219-2228.

Lindsey, M. E., Meyer, M., Thurman, E. M., 2001. Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid phase extraction and liquid chromatography/mass spectrometry. Anal. Chem. 73, 4640-4646.

Lishman, L., Smyth, S. A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland, M., Seto, P., 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Sci. Total Environ. 367, 544-558.

Loeffler, D., Roembke, J., Meller, M., Ternes, T. A., 2005. Environmental fate of pharmaceuticals in water/sediment systems. Environ. Sci. Technol. 39, 5209-5218.

Lotrario, J. B., Stuart, B. J., Lam, T., Arands, R. R., O'Connor, O. A., Kosson, D. S., 1995. Effects of sterilization methods on the physical characteristics of soil: Implications for sorption isotherm analyses. Bull. Environ. Contam. Toxicol., 668-675.

LUA Brandenburg, 2002. Ecotoxicological assessment of human pharmaceuticals in aquatic ecosystems. Eds: Landesumweltamt Brandenburg (LUA). Studien- und agungsberichte 39, 0948-0838.

Marchese, S., Perret, D., Gentili, A., Curini, R., Pastori, F., 2003. Determination of non-steroidal anti-inflammatory drugs in surface water and wastewater by liquid chromatography-tandem mass spectrometry. Chromatographia 58, 263-269.

Margon, A., Pastrello, A., Mosetti, D., Cantone, P., Leita, L., 2009. Interaction between diclofenac and soil humic acids. Soil Sedim. Contam. 18, 489-496.

McArdell, C. S., Molnar, E., Suter, M. J. F., Giger, W., 2003. Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley Watershed, Switzerland. Environ. Sci. Technol. 37, 5479-5486.

Meite, L., Mazellier, P., 2006. Direct photolysis of two pharmaceuticals in aqueous solution. Euro. d'Hydrol. 37, 53-64.

Mendez-Arriaga, F., Esplugas, S., Gimenez, J., 2008. Photocatalytic degradation of non-steroidal anti-inflammatory drugs with TiO_2 and simulated solar irradiation. Water Res. 42, 585-594.

Merck & Co. Inc., 1999. The Merck Manual of Diagnosis and Therapy, Seventeenth Edition.

Merck Index, 1996. An Encyclopedia of Chemicals, Drugs, and Biologicals Twelfth Edition.

Mersmann, P., Scheytt, T., Heberer, T., 2002. Column experiments on the transport behavior of pharmaceutically active compounds in the saturated zone. Acta Hydrochim. Hydrobiol. 30, 275-284.

Meteyer, C. U., Rideout, B. A., Gilbert, M., Shivaprasad, H. L., Oaks, J. L., 2005. Pathology and proposed pathophysiology of diclofenac poisoning in free-living and experimentally exposed Oriental White-Backed Vultures (*Gyps Bengalensis*). Wildl. Dis. 41, 707-716.

Miao, X. S., Koening, B. G., Metcalfe, C. D., 2002. Analysis of acidic drugs in the effluents of sewage treatment plants using liquid chromatography-electrospray ionisation tandem mass spectrometry. J. Chromatogr. A 952, 139-147.

ModelMaker, 2007. www.modelkinetix.com.

Moeder, M., Braun, P., Lange, F., Schrader, S., Lorenz, W. Determination of endocrine disrupting compounds and acidic drugs in water by coupling of derivatization, gas chromatography and negative-chemical ionization mass spectrometry. Clean 2007, 35, 444-451.

Moehle, E., Kempter, C., Kern, A., Metzger, J. W., 1999. Examination of the degradation of drugs in municipal sewage plants using liquid chromatography–electrospray mass spectrometry. Acta Hydrochim. Hydrobiol. 27, 430-436.

Moenickes, S, 2010. Institute of Geoecology, TU Braunschweig. Personal communication.

Moore, D. E., 1998. Mechanisms of photosensitization by phototoxic drugs. Mutat. Res. 422, 165-173.

Moore, D. E., Roberts-Thomson, S., Zhen, D., Duke C. C., 1990. Photochemical studies on the anti-inflammatory drug diclofenac. Photochem. Photobiol. 52, 685-690.

Moore, D. E., 2002. Drug-Induced Cutaneous Photosensitivity: Incidence, mechanism, prevention and management. Drug Saf. 25, 345-372.

Morrison, R. T., Boyd, R. N. (Eds.), 1987. Organic Chemistry, Allyn and Bacon Inc, London, p-1375.

Munoz, I., Gomez-Ramos, M. J., Agueera, A., Garcia-Reyes, J. F., Molina-Diaz A., Fernandez-Alba A. R., 2009. Chemical evaluation of contaminants in wastewater effluents and the environmental risk of reusing effluents in agriculture. Trend. Anal. Chem. 28, 676-694.

Naddeo, V., Meric, S., Kassinos, D., Belgiorno, V., Guida, M., 2009. Fate of pharmaceuticals in contaminated urban wastewater effluent under ultrasonic irradiation. Water Res. 43, 4019-4027.

Naidoo, V., Swan, G. E., 2009. Diclofenac toxicity in Gyps vulture is associated with decreased uric acid excretion and not renal portal vasoconstriction. Comp. Biochem. Physiol., Part C 149, 269-274.

Ng, L. E., Vincent, A. S., Halliwell, B., Wong, K. P., 2006. Action of diclofenac on kidney mitochondrial function. Biochem. Biophys. Res. Commun. 348, 494-500.

Northcott, G. L., Jones, K. C., 2000. Experimental approaches and analytical techniques for determining organic compound bound residues in soil and sediment. Environ. Poll. 108, 19-43.

Nozaki, Y., Lerche, D., Alibo, D. S., Tsutsumi, M., 2000. Dissolved indium and rare earth elements in three Japanese rivers and Tokyo Bay: Evidence for anthropogenic Gd and In. Geochim. Cosmochim. Acta 64, 3975-3982.

Oaks, J. L., Gilbert, M., Virani M. Z., Watson, R. T., Meteyer, C. U., Rideout, B. A., Shivaprasad, H. L., Ahmed, S., Chaudhry, M. J. I., Arshad, M., Mahmood, S., Ali, A., Khan, A. A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630-633.

OECD, 2002a. Guideline for testing of chemicals No. 308. Aerobic and anaerobic transformation in aquatic sediment systems.

OECD, 2002b. Guideline for testing of chemicals No. 106. Adsorption - Desorption using a batch equilibrium method.

Oellers, S., Singer, H. P., Faessler, P., Mueller, S. R., 2001. Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low ng/L level in surface and wastewater. J. Chromatogr. A 911, 225-234.

Oesterreich, T., Klaus, U., Volk, M., Neidhart, B., Spiteller, M., 1999. Environmental fate of amitrole: influence of dissolved organic matter. Chemosphere 38, 379-392.

Onoue, S., Seto, Y., Oishi, A., Yamada S., 2009. Novel methodology for predicting photogenotoxic risk of rharmaceutical substances based on reactive oxygen species (ROS) and DNA-binding assay. Pharm. Sci. 98, 3647-3658.

Osamu, S., Takehiko, M., 2002. Preparation of 2-hydroxycarbazole. Jpn. Kokai Tokkyo Koho 5 pp.

Packer, J. L., Werner, J. J., Latch, D. E., McNeill, K., Arnold, W. A., 2003. Photochemical fate of pharmaceuticals in the environment: Naproxen, diclofenac, clofibric acid, and ibuprofen. Aquat Sci. 65, 342-351.

Paje, M. L., Kuhlicke, U., Winkler, M., Neu, T.R., 2002. Inhibition of lotic biofilms by diclofenac. Appl. Microbiol. Biotechnol. 59, 488-492.

Palomo, M. E., Ballesteros, M. P., Frutos, P., 1999. Analysis of diclofenac sodium and derivatives. Pharm. Biomed. Anal. 21, 83-94.

Pasero, G., Marcolongo, R., Serni, U., Parnham, M. J., Ferrer, F., 1995. A multi-center, double-blind comparative study of the efficacy and safety of aceclofenac and diclofenac in the treatment of rheumatoid arthritis. Curr. Med. Res. Opin. 13, 305-315.

Paxeus, N., 2004. Removal of Selected NSAIDs, gemfibrozil, carbamazepine, ß-blockers, trimethoprim and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment, Water Sci. Technol. 50, 253-260.

Pedersen, J. A., Soliman, M., Suffet, I. H., 2005. Human pharmaceuticals, hormones, and personal care product ingredients in runoff from agricultural fields irrigated with treated wastewater. J. Agric. Food Chem. 53, 1625-1632.

Pedersen, J. A., Yeager, M. A., Suffet, I. H., 2003. Xenobiotic organic compounds in runoff from fields irrigated with treated wastewater. J. Agric. Food Chem. 51, 1360-1372.

Pedrouzo, M., Revert, S., Borrull, F., Pocurull, E., Marc, R. M., 2007. Pharmaceutical determination in surface and wastewaters using high-performance liquid chromatography-(electrospray)-mass spectrometry. Sep. Sci. 30, 297-303.

Perez, S., Barcelo, D., 2008. First evidence for occurrence of hydroxylated human metabolites of diclofenac and aceclofenac in wastewater using QqLIT-MS and QqTOF-MS. Anal. Chem. 80, 8135-8145.

Perez-Estrada, L. A., Malato, S., Gernjak, W., Agueera, A., Thurman, E. M., Ferrer, I., Fernandez-Alba, A. R., 2005. Photo-Fenton degradation of diclofenac: Identification of main intermediates and degradation pathway. Environ. Sci. Technol. 39, 8300-8306.

PharmaDerm prescribtion of Solaraze: http://www.solaraze.com/solaraze/pdf/solaraze_pi.pdf

Philip, J., Szulczewski, D. H., 1973. Photolytic decomposition of N-(2,6-dichloro-m-tolyl) anthranilic acid (meclofenamic acid). Pharm. Sci. 62, 1479-1482.

Phillips, A., Smith, S., Kolpin, D. W., Zaugg, S., Buxton, H., Furlong, E., Eposito, K., Stinson, B., 2010. Pharmaceutical formulation facilities as sources of opioids and other pharmaceuticals to wastewater treatment plant effluents. Environ. Sci. Technol. 44, 4910-4916.

Poiger, T., Buser, H. R., Mueller, M. D., 2001. Photodegradation of the pharmaceutical drug diclofenac in a lake: Pathway, field measurements, and mathematical modeling. Environ. Toxicol. Chem. 20, 256-263.

Poon, G. K., Chen, Q., Teffera, Y., Ngui, J. S., Griffin, P. R., Braun, M. P.,Doss, G. A., Freeden, C., Stearns, R. A., Evans, D. C., Baillie, T. A.,Tang, W., 2001. Bioactivation of diclofenac via benzoquinone imine intermediates identification of urinary mercapturic acid derivatives in rats and humans. Drug Metab. Dispos. 29, 1608-1613.

Poseidon, 2006. Assessment of technologies for the removal of pharmaceuticals and personal care products in sewage and drinking water facilities to improve the indirect Potable Water Reuse. http://poseidon.bafg.de/servlet/is/2888/Final-Report-POSEIDON-May2006.pdf?command=downloadContent&filename=Final-Report-POSEIDON-May2006.pdf

Preuss, G., Willme, U., Zullei-Seibert, N., 2001. Behavior of some pharmaceuticals during artifical groundwater recharge: Elimination and effects on microbiology. Acta Hydrochim. Hydrobiol. 29, 269-277.

Putschew, A., Wischnack, S., Jekel, M., 2000. Occurrence of tri-iodinated X-ray contrast agents in the aquatic environment. Sci. Total Environ. 255, 129-134.

Quintana, J. B., Weiss, S., Reemtsma, T., 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and theiroccurrence in municipal wastewater treated by a membrane bioreactor. Water Res. 39, 2654-2664.

Quintero, B., Miranda, M. A., 2000. Mechanisms of photosensitization induced by drugs: A general survey. Ars. Pharm. 41, 27-46.

Rabiet, M., Togola, A., Brissaud, F., Seidel, J., Budzinski, H., Elbaz-Poulichet, F., 2006. Consequences of treated water recycling as regards pharmaceuticals and drugs in surface and ground waters of a medium-sized Mediterranean catchment. Environ. Sci. Technol. 40, 5282-5288.

Radjenovic J, Petrovic M, Barcelo D., 2007. Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor. Anal. Bioanal. Chem. 387, 1365-1377.

Rafols, C., Roses, M., Bosch, E., 1997. A comparison between different approaches to estimate the aqueous pKa of several non-steroidal anti-inflammatory drugs. Anal. Chim. Acta 338, 127-134.

Ravina, M., Campanella, L., Kiwi, J., 2002. Accelerated mineralization of the drug diclofenac via Fenton reactions in a concentric photoreactor. Water Res. 36, 3553-3560.

Reddersen, K., Heberer, T., 2003. Formation of an artifact of diclofenac during acidic extraction of environmental water samples. J. Chromatogr. A 1011, 221-226.

Richardson, M. L., Bowron, J. M., 1985. The fate of pharmaceutical chemicals in the aquatic environment. Pharm. Pharmacol. 37, 1-12.

Riess, W., Stierlin, H., Degen, P., Faigle, J. W., Gerardin, A., Moppert, J., Sallmann, A., Schmid, K., Schweizer, A., Sulc, M., Theobald, W., Wagner, J., 1978. Pharmacokinetics and metabolism of the anti-inflammatory agent Voltaren. Scand. Rheumatol. Suppl. 22, 17-29.

Roberts, P. H., Thomas, K. V., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Sci. Total Environ. 356, 143-153.

Roennefahrt, I., Koschorreck, J., Kolossa-Gehring, M. (2002): Arzneimittel in der Umwelt. Teil 2: Rechtliche Aspekte und Bewertungskonzepte. Mitt., Umweltchem. Oekotox., 8, (4), 6-9.

Roy, J., Islam, M., Khan, A. H., Das, S C., Akhteruzzaman, M., Deb, A. K., Alam, A, H., 2001. Diclofenac sodium injection sterilized by autoclave and the occurrence of cyclic reaction producing a small amount of impurity. Pharm. Sci. 90, 541-544.

RSMPA, 2004. Report of Swedish Medical Products Agency.

Sacher, F., Lange, F. T., Brauch, H. J., Blankenhorn, I., 2001. Pharmaceuticals in groundwaters. Analytical methods and results of a monitoring program in Baden-Wuerttemberg, Germany. J. Chromatogr. A 938, 199-210.

Sacher, F., Gabriel, S., Metzinger, M., Stretz, A., Wenz, M., Lange, F. T.,Brauch, H. J., Blankenhorn, I., 2002. Occurrence of drugs in ground waters. Results of a monitoring program in Baden-Wuerttemberg. Vom Wasser 99, 183-195.

Sacher, F., Lochow, E., Bethmann, D., Brauch, H. J. 1998. Occurrence of drugs in surface waters. Vom Wasser 90, 233-243.

Sawchuk, R. J., Maloney, J. A., Cartier, L. L., Rackley, R. J., Chan, K. K., Lau, H. S., 1995. Analysis of diclofenac and four of its metabolites in human urine by HPLC. Pharm. Res. 12, 756-762.

Scheurell, M., Franke, S., Shah, R. M., Huehnerfuss, H., 2009. Occurrence of diclofenac and its metabolites in surface water and effluent samples from Karachi, Pakistan. Chemosphere, 77, 870-876.

Scheytt, T., Mersmann, P., Leidig, M., Pekdeger, A., Heberer, T., 2004. Transport of pharmaceutically active compounds in saturated soil columns. Ground Water 42, 767-777.

Scheytt, T., Mersmann, P., Lindstaedt, R., Heberer, T., 2005b. 1-Octanol/water partition coefficients of 5 pharmaceuticals from human medical care: carbamazepine, clofibric acid, diclofenac, ibuprofen, and propyphenazone. Water Air Soil Pollut. 165, 3-11.

Scheytt, T. J., Mersmann, P., Heberer, T., 2006a. Mobility of pharmaceuticals carbamazepine, diclofenac, ibuprofen, and propyphenazone in miscible displacement experiments. Contam. Hydrol. 83, 53-69.

Scheytt, T. J., Mersmann, P., Rejman-Rasinski, E., These, A., 2006b. Tracing pharmaceuticals in the unsaturated zone. J. Soils Sediments 10, 1-10.

Scheytt, T., Mersmann, P., Lindstaedt, R., Heberer, T., 2005a. Determination of sorption coefficients of pharmaceutically active substances carbamazipine, diclofenac, and ibuprofen, in sandy sediments. Chemosphere 60, 245-253.

Schmitt-Jansen, M., Bartels, P., Adler, N., Altenburger, R., 2007. Phytotoxicity assessment of diclofenac and its phototransformation products. Anal. Bioanal. Chem. 387, 1389-1396.

Schneider, W., Degen, P. H., 1981. Simultaneous determination of diclofenac sodium and its hydroxy metabolites by capillary column gas chromatography with electron-capture detection. J. Chromatogr. A 217, 263-271.

Schrey, P., Wilhelm, M., 1999. Drug residues in water samples - findings and their evaluation from the standpoint of drinking water supply. Drug literature study. Part I. Report of the Department of Hygiene and Social and Environmental Medicine of the Ruhr University in Bochum (Germany). DVGW-Schriftenreihe/Wasser 94, 1-70.

Schroeder, H. Fr., 1999. Substance specifc detection and pursuit of non-eliminable compounds during biological treatment of wastewater from the pharmaceutical industry. Waste Manag. 19, 111-123.

Schwaiger, J., Ferling, H., Mallowa, U., Wintermayr, H., Negele, R. D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat. Toxicol. 68,141-150.

SCR PhysProp Database, 2010, http://www.syrres.com/what-we-do/databaseformsaspx?id=386.

Sebastine, I., Wakeman, R., 2003. Consumption and environmental hazards of pharmaceutical substances in the UK. Process Saf. Environ. Protect. 81, 229-235.

Sebok, A., Vasanits-Zsigrai, A., Palko, G., Zaray, Gy., Molnar-Perl, I., 2008. Identification and quantification of ibuprofen, naproxen, ketoprofen and diclofenac present in waste-waters, as their trimethylsilyl derivatives, by gas chromatography mass spectrometry. Talanta 76, 642-650.

Shen, S., Marchick, M. R., Davis, M. R., Doss, G. A., Pohl, L. R., 1999. Metabolic activation of diclofenac by human cytochrome P450 3A4: Role of 5-hydroxydiclofenac. Chem. Res. Toxicol. 12, 214-222.

Silva, A. R. M., Portugal F. C. M., Nogueira, J. M. F., 2008. Advances in stir bar sorptive extraction for the determination of acidic pharmaceuticals in environmental water matrices: Comparison between polyurethane and polydimethylsiloxane polymeric phases. J. Chromatogr. A 1209, 10-16.

Spadotto, C. A., Hornsby. A. G., 2003. Soil sorption of acidic pesticides: Modeling pH effects. Environ. Qual. 32, 949-956.

Stierlin, H., Faigle, J. W., Sallmann, A., Kueng, W., Richter, W. J., Kriemler, H. P., Alt, K. O., Winkler, T., 1979. Biotransformation of diclofenac sodium (Voltaren) in animals and in man. I. Isolation and identification of principal metabolites. Xenobiotica 9, 601-610.

Strenn, B., Clara, M., Gans, O., Kreuzinger, N., 2004. Carbamazepine, diclofenac, ibuprofen and bezafibrate: Investigations on the behavior of selected pharmaceuticals during wastewater treatment. Water Sci. Technol. 50, 269-276.

Stuelten, D., Lamshoeft, M., Zuehlke, S., Spiteller, M., 2008a. Isolation and characterization of a new human urinary metabolite of diclofenac applying LC-NMR-MS and high resolution mass analyses. Pharmaceut. Biomed. 47, 371-376.

Stuelten, D., Zuehlke, S., Lamshoeft, M., Spiteller, M., 2008b. Occurrence of diclofenac and selected metabolites in sewage effluents. Sci. Total Environ. 405, 310-316.

Stuer-Lauridsen, F., Birkved, M., Hansen, L. P., Holten-Luetzhoft, H. C., Halling-Sørensen, B., 2000. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. Chemosphere 40, 783-793.

Stumpf, M., Ternes, T. A., Haberer, K., Seel, P., Baumann, W., 1996. Determination of pharmaceutics in sewage plants and river water. Vom Wasser 86, 291-303.

Stumpf, M., Ternes, T. A., Wilken, R. D., Rodrigues, S. V., Baumann, W., 1999. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. Sci. Total. Environ. 225, 135-141.

Suarez, S., Carballa, M., Omil, F., Lema, J. M., 2008. How are pharmaceutical and personal care products (PPCPs) removed from urban wastewaters? Rev. Environ. Sci. Biotechnol. 7, 125-138.

Taggart, M. A., Cuthbert, R., Das, D., Sashikumar, C., Pain, D. J., Green, R. E., Feltrer, Y., Shultz, S., Cunningham, A. A., Meharg, A. A., 2007a. Diclofenac disposition in Indian cow and goat with reference to Gyps vulture population declines. Environ. Poll. 147, 60-65.

Taggart, M. A., Senacha, K. R., Green, R. E., Jhala, Y. V., Raghavan, B., Rahmani, A. R., Cuthbert, R., Pain, D. J., Meharg, A. A., 2007b. Diclofenac residues in carcasses of domestic ungulates available to vultures in India. Environ. Int. 33, 759-765.

Tauxe-Wuersch, A., De Alencastro, L. F., Grandjean, D., Tarradellas, J., 2005. Occurrence of several acidic drugs in sewage treatment plants in Switzerland and risk assessment. Water Res. 39, 1761-1772.

Ternes, T. A, Stumpf, M., Mueller, J., Haberer, K., Wilken, R. D., Servos, M., 1999. Behavior and occurence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225, 81-90.

Ternes, T. A., 1998, Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245-3260.

Ternes, T. A., Hirsch, R., 2000. Occurrence and behavior of X-ray contrast media in sewage facilities and the aquatic environment. Environ. Sci. Technol. 34, 2741-2748.

Ternes, T. A., 2001. Pharmaceuticals in surface waters. Wasser & Boden 53, 9-14.

Ternes, T., Meisenheimer, M., McDowell, D., Sacher, F., Brauch, H. J., Haist-Gulde, B., Preuss, G., Willme, U., Zullei-Seibert, N., 2002. Removal of pharmaceuticals during drinking water treatment. Environ. Sci. Technol. 36, 3855-3863.

Ternes, T., Herrmann, N., Bonerz, M., Knacker, T., Siegrist, H., Joss, A., 2004. A rapid method to measure the solid-water distribution coefficient (K_d) for pharmaceuticals and musk fragrances in sewage sludge. Water Res. 38, 4075-4084.

Terzic, S., Senta, I., Ahel, M., Gros, M., Petrovic, M., Barcelo, D., Mueller, J., Knepper, T., Marti, I., Ventura, F., Jovancic, P., Jabucar, D., 2008. Occurrence and fate of emerging wastewater contaminants in Western Balkan Region. Sci. Total Environ. 399, 66-77.

Thomas, P. M., Forster, G. D., 2005. Tracking acidic pharmaceuticals, caffeine, and triclosan through the wastewater treatment process. Environ. Toxicol. Chem. 24, 25-30.

Thomas, P. M., Foster, G. D., 2004. Determination of nonsteroidal anti-inflammatory drugs, caffeine, and triclosan inwastewater by gas chromatography–mass spectrometry. Environ. Sci. Health. 39, 1969-1978.

Tixier, C., Singer, H. P., Oellers, S., Mueller, S. R., 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. Environ. Sci. Tech. 37, 1061-1068.

Togola, A, Budzinski, H., 2007. Analytical development for analysis of pharmaceuticals in water samples by SPE and GC/MS. Anal. Bioanal. Chem. 388, 627-635.

Togola, A., Budzinski, H., 2008. Multi-residue analysis of pharmaceutical compounds in aqueous samples. J. Chromatogr. A 1177, 150-158.

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Koehler H-R., Schweiger, J., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhyncus mykiss*), Aquat. Toxicol. 68, 151-166.

Triebskorn, R., Casper, H., Scheil, V., Schwaiger, J., 2007. Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Anal. Bioanal. Chem. 387, 1405-1416.

Tudja, P., Khan, M. Z., Mestrovic, E., Horvat, M., Golja, P., 2001. Thermal behavior of diclofenac sodium: decomposition and melting characteristics. Chem. Pharmaceut. Bull. 49, 1245-1250.

Van Wezel, A. P., Jager, T., 2002. Comparison of two screening level risk assessment approaches for six disinfectants and pharmaceuticals. Chemosphere 47, 1113-1128.

Vieno, N. M., Harkki, H., Tuhkanen, T., Kronberg, L., 2007. Occurrence of pharmaceuticals in river water and their elimination in a pilot-scale drinking water treament plant. Environ. Sci. Technol., 5077-5084.

Vieno, N. M., Tuhkanen, T., Kronberg, L., 2005. Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. Environ. Sci. Technol. 39, 8220-8226.

Vogna, D., Marotta, R., Napolitano, A., Anderozzi, R., d'Ischia, M., 2004. Advanced oxidation of the pharmaceutical drug diclofenac with UV/H_2O_2 and ozone. Water Res. 38, 414-422.

Waggot, A., 1981. Trace organic substances in the River Lee (Great Britain). Chem. Water Reuse. 2, 55-99.

Webster, R., Pacey, M., Winchester, T., Johnson, P., Jezequel, S., 1998. Microbial oxidative metabolism of diclofenac: production of 4'-hydroxydiclofenac using Epiccocum nigrum IMI354292. Appl. Microbiol. Biotechnol. 49, 371-376.

Werres, F., Stien, J., Balsaa, P., Schneider, A., Winterhalter, P., Overath, H., 2000. Automated determination of polar residues from pharmaceutical products in the aquatic system using solid-phase microextraction and derivatization. Vom Wasser, 94, 135-147.

Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., 2005. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. Environ. Sci. Technol. 39, 6649-6663.

Wiegel, S., Aulinger, A., Brockmeyer, R., Harms, H., Loeffler, J., Reincke, H., Schmidt, R., Stachel, B., von Tuempling, W., Wanke, A., 2004. Pharmaceuticals in the river Elbe and its tributaries. Chemosphere 57, 107-126.

Wiesenberg-Boettcher, I., Pfeilschifter, J., Schweizer, A., Sallmann, A., Wenk, P. 1991. Pharmacological properties of five diclofenac metabolites identified in human plasma. Agents Actions 34, 135-137.

Woldegiorgis, A., Green, J., Remberger, M., Kaj, L., Brorstroem-Lunden, E., Dye, C., Schlabach, M., 2007. Results from the Swedish screening 2006: Sub report 4: Pharmaceuticals. IVL Report (B1751), A, B 1-73.

WHO, 2002. World Health Organization. Wildlife. In global assessment on the State of the Science of Endocrine Disruptors. Damstra, T., et al., Eds.; WHO/PCS/EDC/02.2 Chapter 4.

Zajac, M., Stanisz, B., Musial, W., 1998. The stability of diclofenac in acidic medium. Acta Pol. Pharm. 55, 371-374.

Zhang, Y., Geißen, S. U., Gal, C., 2008. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73, 1151-1161.

Zilnik, L. F., Jazbinsek, A., Hvala, A., Vrecer, F., Klamt, A., 2007. Solubility of sodium diclofenac in different solvents. Fluid Phase Equilibria 261, 140-145.

Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. Lancet 355,1789-1790.

Zuccato, E., Castiglioni, S., Fanelli, R., 2005. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. Hazard. Mat. 122, 205-209.

Zuehlke, S., Duennbier, U., Heberer, T., 2004. Determination of polar drug residues in sewage and surface water applying liquid chromatography-tandem mass spectrometry. Anal. Chem. 76, 6548-6655.

Zwiener, C., Gremm, T. J., Frimmel, F. H., 2001. Pharmaceutical residues in the aquatic environment and their significance for drinking water production. In: Kuemmerer, K., editor. Pharmaceuticals in the Environment. Berlin: Springer, pp 201-211.

Zwiener, C., Frimmel, F. H., 2003, Short-term tests with a pilot sewage plant and biofilm reactors for the biological degradation of the pharmaceutical compounds clofibric acid, ibuprofen, and diclofenac. Sci. Total Environ. 309, 201-211.

Zwiener, C., Frimmel, H., 2000. Oxidative treatment of pharmaceuticals in water. Water Res., 34, 1881-1885.

Appendix

Table A1: Extraction efficiency tests of SPE materials for ^{14}C-diclofenac from pure water

	A [Bq] (fortified in the water phase)	A [Bq] (found in the methanol eluent after SPE)	Recovery [%]
Sample 1	7000	6742	96
Sample 2	7000	6542	93
Sample 3	7000	6565	93
Sample 3	7000	6240	89
Mean			**93**
SD			**3**
RSD			3.2

A = radioactivity amount

Table A2: Extraction efficiency of ethyl acetate for diclofenac from pure water as determined by radiotracer technique

	Fortified [Bq]	Found [Bq]	Recovery [%]
Sample1	35647	33980	95
Sample 2	35647	33152	93
mean			**94**
SD			**1.6**

Table A3: Extraction efficiency of ethyl acetate for diclofenac from pure water as determined by GC/MS

	Fortified [ng/µL]	Found [ng/µL]	Recovery [%]
Sample1	10	8.8	88
Sample 2	10	9.0	90
mean			**89**
SD			**1.4**

Table A4: Extraction efficiency of ethyl acetate for diclofenac from pure water as determined by HPLC/UVD

	Fortified [ng/µL]	Found [ng/µL]	Recovery [%]
Sample 1	100	93.1	93.1
Sample 2	100	89.0	89.0
Sample 3	100	91.6	91.6
Mean			**92.0**
SD			**2.2**
RSD			**2.4**

Table A5: Extraction efficiency of ethyl acetate for diclofenac from pure water as determined by LC/MS/MS

	Fortified [ng/µL]	Found [ng/µL]	Recovery [%]
Sample 1	10	8.77	87.7
Sample 2	10	9.00	90.0
Sample 3	10	8.90	89.0
Mean			**88.9**
SD			**1.2**
RSD			1.4

Table A6: Test on solvent exchange step from ethyl acetate to methanol as determined by HPLC/UVD

	[ng/µL] fortified from ethyl acetate solution	[ng/µL] found after dissolution in methanol	Recovery [%]
Sample 1	50	49.3	98.5
Sample 2	50	49.5	99.0
Sample 3	50	51.6	103.2
Mean			**100.2**
SD			**2.6**
RSD			2.6

Table A7: Recovery rates of diclofenac after extraction with ethyl acetate from wet sediment and clean up with GPC as determined by HPLC/UVD

	Fortified [ng/µL]	Found [ng/µL]	Recovery [%]
Sample 1	40	40.5	101.3
Sample 2	40	39.9	99.6
Sample 3	40	38.8	97.1
Sample 4	40	40.6	101.5
Sample 5	80	71.3	89.1
Mean			**97.7**
SD			**5.1**
RSD			5.3

Table A8: Calibration curve parameters for the determination of diclofenac concentrations

	Linearity range	Regression equation	R^2
GC/MS (DCF-PFB ester)	0.3 - 30 ng/µL	PA x 10^{-5} = 2.70 C + 1.30	0.997
GC/MS (DCF)	1.0 - 10 ng/µL	PA x 10^{-4} = 3.30 C – 3.00	0.997
HPLC/UVD	0.2 - 100 ng/µL	PA x 10^{-5} = 2.20 C + 1.30	0.999
LC/MS/MS	0.0001 - 10 ng/µL	PA x 10^{-3} = 7.74 C + 736	0.995
LC/MS/MS	1.0 - 20 ng/µL	PA x 10^{-6} = 4.00 C + 4.00	0.996

PA = a ± bc (PA, peak area; a, intercept; b, slop), DCF = diclofenac, DCF-PFB ester = diclofenac pentafluorobenzyl ester

Table A9: Determination of sorption coefficient (K_d) at different concentrations

Set	C_w Bq/60mL	C_w Bq/L	C_w µg/L	Log C_w µg/L	C_s Bq/20g	C_s Bq/kg	C_s µg/kg	Log C_s µg/kg	K_d (L/kg) n = 4
S 1	3732	62207	8.4	**0.9**	7241	362030	48.8	**1.7**	**5.8 ± 0.8**
S 2	6192	103202	13.9	**1.1**	11755	587744	79.3	**1.9**	**5.7 ± 0.7**
S 3	14699	244993	33.0	**1.5**	21876	1093821	147.5	**2.2**	**4.5 ± 1.0**
S 4	30532	508875	68.6	1.9	24332	1216600	164.1	2.2	2.4 ± 0.1
S 5	23591	393190	53.0	**1.7**	49561	2478030	334.2	**2.5**	**6.4 ± 1.3**
S 6	36378	606294	81.8	**1.9**	59094	2954717	398.5	**2.6**	**4.9 ± 0.5**
Mean									**5.5 ± 0.8**

S = set, C_s = sorbed concentrations, C_w = aqueous concentrations, n = number of replicates

Table A10 : Determination of desorption coefficient (K_{des}) at different concentrations

Set	C_w Bq/ 60 mL	Cw Bq/L	C_w µg/L	Log C_w µg/L	Cs Bq/20g	Cs Bq/kg	C_s µg/kg	Log C_s µg/kg	K_{des} (L/kg) n = 4
S 1	nd	nd	nd	**nd**	nd	nd	nd	**nd**	**nd**
S 2	2652	44202	6.0	**0.7**	9103	455139	61.4	**1.8**	**10.2 ± 1.7**
S 3	3447	57457	7.7	**0.9**	18429	921449	124.3	**2.1**	**16.1 ± 2.5**
S 4	5594	93240	12.6	**1.1**	18738	936879	126.3	**2.1**	**10.0 ± 1.9**
S 5	8794	146571	19.8	**1.3**	40766	2038317	274.9	**2.4**	**13.9 ± 0.5**
Mean									**13.0 ± 3.0**

S = set, C_s = sorbed concentrations, C_w = aqueous concentrations, n = number of replicates

Table A11: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the water phase of water/sediment system WS1 after incubation in the dark as shown by solvent 1 (n-hexane/ethyl acetate/acetic acid; 50:50:1 v/v/v)

Laboratory batch system						
DAA	**DCF [%]**	**BTP1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**
0	81.5	3.7	3.8	7.4	1.6	7.5
1	66.1	3.2	3.5	4.4	6.4	2.0
3	62.5	2.6	nd	nd	8.2	3.5
7	43.6	3.3	0.9	1.3	12.1	9.7
14	43.5	4.1	nd	nd	13.0	9.5
28	1.3	13.9	3.2	nd	4.0	10.0
56	1.6	14.2	1.5	nd	1.7	nd
100	0.5	9.4	0.6	nd	0.2	nd
Biometric flask system						
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**
3	61.2	2.4	nd	nd	9.0	4.2
56	1.5	16.2	1.6	nd	1.4	0.4
100	0.9	9.8	1.0	nd	0.2	0.3

DCF = diclofenac, BTP = biotransformation product

Table A12: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the water phase of water/sediment system WS1 after incubation in the dark as shown by solvent 2 (dichloromethane/methanol/25% ammonia; 85:14:1; v/v/v)

Laboratory batch system					
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**
0	80.4	7.8	nd	3.1	14.2
1	60.0	5.6	nd	1.8	18.2
3	65.5	4.3	nd	nd	7.0
7	48.6	7.1	2.9	nd	12.3
14	61.5	2.8	nd	nd	5.8
28	15.0	13.5	2.2	0.3	1.4
Biometric flask system					
3	69	3	nd	nd	4.8

Table A13: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the water phase of water/sediment system WS2 after incubation in the dark as shown by solvent 1 (n-hexane/ethyl acetate/acetic acid; 50:50:1 v/v/v)

Laboratory batch system							
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**	**BTP 6 [%]**
0	77.3	3.40	8.60	8.40	1.60	6.30	Nd
1	65.1	2.30	4.00	5.30	6.50	3.20	Nd
3	38.3	2.50	2.60	nd	8.30	23.10	nd
7	36.1	3.10	3.20	nd	6.50	15.20	nd
14	35.4	4.50	1.90	nd	6.60	3.10	nd
28	20.8	4.20	1.30	1.00	6.00	0.60	nd
56	5.20	7.34	nd	nd	3.70	0.86	1.36
100	0.74	6.63	0.19	nd	nd	nd	1.49
Biometric flask system							
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**	**BTP 6 [%]**
3	54.80	1.70	0.90	2.00	6.70	7.60	nd
56	4.95	7.10	nd	nd	4.29	1.76	1.32
100	1.69	6.86	nd	nd	1.12	0.10	1.70

Table A14: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the water phase of water/sediment system after WS2 incubation in the dark as shown by solvent 2 (dichloromethane/methanol/25% ammonia; 85:14:1; v/v/v)

Laboratory batch system					
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**
0	83.70	7.30	14.60	nd	nd
1	73.00	4.70	8.70	nd	nd
3	37.90	3.10	33.80	nd	nd
7	38.40	3.60	16.70	nd	nd
14	37.70	8.00	5.80	nd	nd
28	22.60	8.00	1.60	nd	1.7
56	9.20	6.80	2.46	nd	nd
100	1.60	7.25	0.20	nd	nd
Biometric flask system					
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**
3	67.80	3.60	2.30	nd	nd
56	9.78	6.26	2.78	0.60	nd
100	1.70	8.10	1.40	0.27	nd

Table A15: Extraction efficiency test for ^{14}C-diclofenac in wet sediment using different solvents

Type of solvent	ER [%]	NER [%]	Recovery [%]
Ethyl acetate	**90 ± 3.5**	9 ± 0.6	99 ± 4.0
Acetone	**69 ± 4.7**	25 ± 2.2	94 ± 2.4
Acetonitrile	**69 ± 3.6**	21 ± 4.6	90 ± 3.0
Methanol	**57 ± 2.9**	18 ± 3.6	75 ± 2.0

Table A16: Tests on remaining radioactivity of ^{14}C-diclofenac in pore water after direct solvent extraction

Method of extraction	MIN [%]	AP [%]	ER [%]	NER [%]	Recovery [%]
DE	1 ± 0.02	41 ± 0.5	40 ± 2.4	11 ± 4.6	93 ± 1.7
RE	1 ± 0.02	53 ± 0.0	30 ± 0.9	15 ± 3.1	99 ± 2.2

DE = direct extraction, RE = Rinsing before extraction

Table A17: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the ethyl acetate extracts of water/sediment system WS1 after incubation in the dark as shown by solvent 1 (n-hexane/ethyl acetate/acetic acid; 50:50:1 v/v/v)

Laboratory batch system								
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**	**BTP 6 [%]**	**BTP 7 [%]**
1	6.20	0.25	1.50	0.50	nd	nd	nd	nd
3	9.07	0.38	2.95	1.20	nd	nd	nd	nd
7	9.70	0.33	2.47	0.85	2.40	nd	nd	nd
14	10.00	0.18	2.79	0.50	2.30	nd	nd	nd
28	2.05	0.48	2.62	0.28	1.22	0.20	0.36	0.35
Biometric flask system								
3	8.63	0.45	3.69	1.31	0.83	nd	nd	nd

Table A18: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the ethyl acetate extracts of water/sediment system WS1 after incubation in the dark as shown by solvent 2 (dichloromethane/methanol/25% ammonia; 85:14:1; v/v/v)

Laboratory batch system								
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**	**BTP 6 [%]**	**BTP 7 [%]**
1	6.35	0.50	0.30	0.80	0.50	nd	nd	nd
3	10.75	0.62	0.62	1.11	0.50	nd	nd	nd
7	13.27	0.29	0.42	1.27	0.50	nd	nd	nd
14	14.34	nd	nd	1.42	nd	nd	nd	nd
28	4.99	0.38	nd	1.54	nd	nd	0.21	0.44
Biometric flask system								
3	11.85	0.80	0.54	1.00	0.72	nd	nd	nd

Table A19: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the ethyl acetate extracts of water/sediment system WS2 after incubation in the dark as shown by solvent 1 (n-hexane/ethyl acetate/acetic acid; 50:50:1 v/v/v)

Laboratory batch system				
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**
1	10.4	0.2	2.9	1.4
3	13.0	0.2	3.3	2.4
7	16.0	0.3	4.0	1.9
14	14.4	0.6	5.0	1.9
28	6.4	0.9	4.4	1.6
56	1.0	0.8	2.3	0.6
Biometric flask system				
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**
3	14.0	0.3	3.7	2.5

Table A20: Relative radioactivity amounts of ^{14}C-DCF and its BTPs in the ethyl acetate extracts of water/sediment system WS2 after incubation in the dark as shown by solvent 2 (dichloromethane/methanol/25% ammonia; 85:14:1; v/v/v)

Laboratory batch system							
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**	**BTP 6 [%]**
1	11.4	0.6	0.7	0.2	0.3	0.7	1.0
3	14.6	0.3	0.6	0.1	0.3	1.3	1.7
7	20.0	0.3	nd	nd	nd	0.7	1.2
14	19.4	0.6	nd	nd	nd	0.5	1.4
28	11.1	0.5	nd	nd	nd	0.3	1.4
56	3.6	0.4	nd	nd	nd	nd	0.7
Biometric flask system							
DAA	**DCF [%]**	**BTP 1 [%]**	**BTP 2 [%]**	**BTP 3 [%]**	**BTP 4 [%]**	**BTP 5 [%]**	**BTP 6 [%]**
3	16.0	0.5	0.6	nd	0.3	1.4	1.7
56	2.7	0.4	nd	nd	nd	nd	0.5

Table A21: Balances of the biotransformation test series of ^{14}C-diclofenac in water/sediment system WS1 using laboratory batch system

DAA	MIN [%]	AP [%]	ER [%]	NER [%]	Recovery [%]
0	nd	106 ± 0.5	0.7 ± 0.1	2.0 ± 0.1	109 ± 0.5
1	0.1 ± 0.0	86 ± 0.2	9.0 ± 1.0	13.0 ± 2.4	109 ± 3.6
3	0.4 ± 0.1	77 ± 2.0	14.0 ± 1.0	17.0 ± 1.3	108 ± 2.1
7	0.8 ± 0.0	71 ± 0.3	16.0 ± 1.4	21.0 ± 3.5	108 ± 1.8
14	1.0 ± 0.0	70 ± 1.0	16.0 ± 2.6	21.0 ± 2.8	108 ± 0.7
28	3.0 ± 0.5	32 ± 3.0	8.0 ± 0.1	63.0 ± 1.6	106 ± 3.7
56	6.0 ± 0.2	19 ± 1.0	4.0 ± 0.3	76.0 ± 0.2	106 ± 1.8
100	13.0 ± 0.2	10 ± 1.0	0.2 ± 0.0	83.0 ± 0.0	106 ± 0.7

DAA = Days after application

Table A22: Balances of the biotransformation test series of ^{14}C-diclofenac in water/sediment system WS2 using laboratory batch system

DAA	MIN [%]	AP [%]	ER [%]	NER [%]	Recovery [%]
0	nd	106 ± 1.5	0.8 ± 0.1	2 ± 0.4	109 ± 1.7
1	0.1 ± 0.0	86 ± 1.1	15.0 ± 1.0	8 ± 0.2	109 ± 2.3
3	0.1 ± 0.1	75 ± 1.0	19.0 ± 2.7	14 ± 0.2	109 ± 4.0
7	0.5 ± 0.0	64 ± 0.8	22.0 ± 0.5	25 ± 0.4	112 ± 1.8
14	0.9 ± 0.1	52 ± 1.2	22.0 ± 1.0	32 ± 3.7	107 ± 1.3
28	3.0 ± 0.2	34 ± 0.1	13.0 ± 0.6	55 ± 2.6	105 ± 3.1
56	6.0 ± 0.7	19 ± 0.7	5.0 ± 2.8	77 ± 1.1	107 ± 0.0
100	13.0 ± 0.1	9 ± 0.2	0.8 ± 0.1	82 ± 0.1	105 ± 0.2

Table A23: Balances of the biotransformation test series of ^{14}C-diclofenac in water/sediment system WS1 using the biometric flask system

DAA	MIN [%]	AP [%]	ER [%]	NER [%]	Recovery [%]
3	0.6 ± 0.0	77 ± 2.8	15.0 ± 0.2	16 ± 7.0	108 ± 3.9
56	6.0 ± 0.0	21 ± 1.5	4.0 ± 0.1	75 ± 0.5	106 ± 0.0
100	13.0 ± 3.5	12 ± 1.8	0.4 ± 0.1	82 ± 0.5	107 ± 2.0

Table A24: Balances of the biotransformation test series of ^{14}C-diclofenac in water/sediment system WS2 using the biometric flask system

DAA	MIN [%]	AP [%]	ER [%]	NER [%]	Recovery [%]
3	0.2 ± 0.0	74 ± 3.4	21 ± 2.8	13 ± 1.6	108 ± 7.8
56	5.0 ± 3.6	19 ± 0.7	4 ± 2.0	75 ± 0.3	103 ± 6.5
100	13 ± 1.8	12 ± 1.3	1 ± 0.8	82 ± 4.5	108 ± 4.8

Table A25: Balances of the phototransformation test of ^{14}C-diclofenac irradiated 10 h/d for three days using laboratory batch systems

	MIN [%]	AP [%]	ER [%]	NER [%]	Recovery [%]
WS1	1 ± 0.2	35 ± 4.2	3 ± 0.7	65 ± 1.8	104 ± 2.8
WS2	1 ± 0.3	26 ± 3.8	10 ± 0.6	70 ± 0.1	107 ± 4.7
QS	1 ± 0.0	66 ± 0.0	10 ± 0.0	19 ± 0.0	96 ± 0.0

Table A26: Relative radioactivity amounts of ^{14}C-DCF and its PTPs after irradiation of ^{14}C-DCF 10 h/d for three days using laboratory batch systems

Water/sediment 1								
	DCF [%]	**PTP 1 [%]**	**PTP 2 [%]**	**PTP 3 [%]**	**PTP 4 [%]**	**PTP 5 [%]**	**PTP 6 [%]**	**PTP 7 [%]**
AP a	2.00	29.00	1.00	4.70	nd	nd	nd	nd
AP b	nd	36.70	nd	nd	nd	nd	nd	nd
ER a	1.22	0.28	0.32	0.28	0.63	0.27	0.13	0.10
ER b	1.96	0.20	0.16	0.71	0.20	nd	nd	nd
Water/sediment 2								
	DCF [%]	**PTP 1 [%]**	**PTP 2 [%]**	**PTP 3 [%]**	**PTP 4 [%]**	**PTP 5 [%]**	**PTP 6 [%]**	**PTP 7 [%]**
AP a	2.2	15.9	2.7	2.5	0.1	0.2	2.3	nd
AP b	4.0	17.9	1.5	nd	nd	nd	nd	nd
ER a	nd							
ER b	nd							
Pure water/Quartz sand sediment								
	DCF [%]	**PTP 1 [%]**	**PTP 2 [%]**	**PTP 3 [%]**	**PTP 4 [%]**	**PTP 5 [%]**	**PTP 6 [%]**	**PTP 7 [%]**
AP a	7.0	45.0	6	15	nd	nd	nd	nd
ER a	nd							
Water samples								
	DCF [%]	**PTP 1 [%]**	**PTP 2 [%]**	**PTP 3 [%]**	**PTP 4 [%]**	**PTP 5 [%]**	**PTP 6 [%]**	**PTP 7 [%]**
NW a	3.5	63.75	3.35	6.00	nd	nd	nd	nd
PW a	nd	73.00	5.10	16.08	11.4	nd	nd	nd

AP = aqueous phase, ER = ethyl acetate extract, NW = Native water, PW = Pure water, a = solvent 1, b = solvent 2, DCF = diclofenac, PTP = phototransformation product, nd = not determined

Table A27: Relative radioactivity amounts of ^{14}C-DCF and its TPs detected in the percolate and methanol eluent after SPE

Laboratory batch system (dark experiments)				
DAA	**WS1**		**WS2**	
	P	**ME**	**P**	**ME**
0	2.9 ± 0.6	80.6 ± 2.0	4.2 ± 0.5	79.6 ± 3.6
1	1.7 ± 0.1	60.9 ± 0.8	1.9 ± 0.3	76.5 ± 0.8
3	1.2 ± 0.1	63.0 ± 1.8	1.4 ± 0.0	67.6 ± 0.8
7	1.0 ± 0.1	63.5 ± 0.2	1.0 ± 0.1	58.7 ± 0.8
14	1.0 ± 0.0	63.0 ± 0.6	1.2 ± 0.1	41.4 ± 0.2
28	2.3 ± 0.2	23.9 ± 1.8	0.7 ± 0.2	25.6 ± 1.4
56	1.4 ± 0.1	12.7 ± 0.9	0.7 ± 0.0	12.7 ± 2.9
100	1.0 ± 0.3	6.7 ± 0.6	0.7 ± 0.0	6.1 ± 0.1
Biometric flask system				
3	1.3 ± 0.3	59.0 ± 2.0	1.3 ± 0.0	55.6 ± 2.4
56	1.5 ± 0.2	12.7 ± 2.8	0.9 ± 0.3	14.6 ± 1.0
100	0.7 ± 0.2	7.8 ± 0.9	0.7 ± 0.2	8.1 ± 1.0
Laboratory batch system (Irradiation experiments)				
3	3.4 ± 0.1	19.4 ± 5.2	2.0 ± 0.5	15.7 ± 3.0

P = percolate, ME = methanol eluent, TPs = transformation products,

Table A28: Chemical characterization of the non-extractable residues of WS1 by means of sequential extraction technique after the 3-d irradiation experiment

		Radioactivity [%]
MIN		**0.9 ± 0.2**
AP		**35.0 ± 4.2**
Ethyl acetate	ER	**3.0 ± 0.7**
	NER	64.6 ± 1.8
Methanol/ Hydrochloric acid	ER	**9.0 ± 1.3**
	NER	51.0 ± 7.0
Dimethylformamide/ Chlorotrimethylsilane	ER	**26.0 ± 0.4**
	NER	**26.0 ± 1.7**
Recovery [%]		**100.0 ± 6.4**

Table A29: Concentrations of irradiated diclofenac in pure water as determined by GC/MS

Time (h)	**Fortified [ng/µL]**	**Found [ng/µL]**	**Recovery [%]**
1	10.00	5.72	**57.20**
2	10.00	4.77	**47.70**
3	10.00	3.22	**32.20**
4	10.00	3.14	**31.40**
5	10.00	2.09	**20.90**

Table A30: Concentrations of irradiated diclofenac in pure water as determined by HPLC/UVD

Time (h)	Fortified [ng/µL]	Found [ng/µL]	Recovery [%]
2	100	32.7	**32.7**
4	100	21.7	**21.7**
6	100	12.8	**12.8**
8	100	7.8	**7.8**
10	100	3.7	**3.7**

Table A31: Concentrations of irradiated diclofenac in pure water as determined by LC/MS/MS

Time (h)	Fortified [ng/µL]	Found [ng/µL]	Conc [ng/µL] (Dilution factor = 2)	Recovery [%]
2	100	17.2	34.4	**34.4**
4	100	11.5	23.0	**23.0**
6	100	6.8	13.6	**13.6**
8	100	4.5	9.0	**9.0**
10	100	2.2	4.4	**4.4**

Table A32: Concentrations of irradiated diclofenac in real water/sediment system WS3 as determined by LC/MS/MS

Water phase			
	Fortified [ng/µL]	**Found [ng/µL]**	**Recovery [%]**
Sample 1	20	7.63	38.15
Sample 2	20	8.08	40.40
Mean			**39.30**
SD			**1.60**
RSD			4.10
Ethyl acetate extracts			
Sample 1	20	0.516	2.6
Sample 2	20	0.457	2.3
Mean			**2.4**
SD			**0.2**
RSD			8.6

Table A33: Concentrations of diclofenac in real water/sediment system WS3 as determined by LC/MS/MS after the dark experiments

Water phase			
	Fortified [ng/µL]	**Found [ng/µL]**	**Recovery [%]**
Sample 1	20	11.6	58.0
Sample 2	20	12.1	60.5
Mean			**59.3**
SD			**1.8**
RSD			3.0
Ethyl acetate extracts			
Sample 1	20	3.90	19.5
Sample 2	20	3.79	19.0
Mean			**19.2**
SD			**0.4**
RSD			2.0

Curriculum Vitae

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